UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS
DEPARTAMENTO DE QUÍMICA ORGÁNICA

TESIS DOCTORAL

Producción y optimización de bioplaguicidas de *Artemisia absinthium y Lavandula luisieri*

Production and optimization of biopesticides from *Artemisia absinthium and Lavandula luisieri*

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Luis Fernando Julio Torres

DIRECTORES

Azucena González Coloma
Carmen Elisa Díaz Hernández
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CSIC
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Memoria que para optar al grado de

DOCTOR

Presenta

Luis Fernando Julio Torres

MADRID, 2015

La Dra. Azucena González Coloma, Investigadora Científica del Consejo Superior de Investigaciones Científicas, la Dra. Carmen Elisa Díaz Hernández, Científico Titular del Consejo Superior de Investigaciones Científicas y la Dra. María Fe Andrés Yeves, Científico Titular del Consejo Superior de Investigaciones Científicas.

Autorizan:

La presentación de la Memoria Titulada "**Producción y Optimización de Bioplaguicidas de *Artemisia absinthium* y *Lavandula luisieri***" ("Production and optimization of Biopesticides from *Artemisia absinthium* and *Lavandula luisier*"), que ha sido realizada en el Instituto de Ciencias Agrarias (ICA-CSIC), dentro del período 2010-2015, bajo nuestra dirección, dado que reúne la cantidad y calidad de trabajo necesaria para constituir la Tesis Doctoral, que el Licenciado en Química Luis Fernando Julio Torres presenta para optar al grado de Doctor por la Facultad de Ciencias Químicas de la Universidad Complutense de Madrid en el Programa de Doctorado de "Química Orgánica", cumpliendo los requisitos exigidos por esta Universidad para optar a la Mención Europea.

Dra. Azucena González Coloma

Dra. Carmen Elisa Díaz Hernández

Dra. María Fe Andrés Yeves

Madrid, Noviembre de 2015

A mis padres Guillemo A. Julio y Sol Maria Torres
A mis hermanos Ana I, Jorge L. y Liseth Julio
A mis sobrinos Valeria, Jeremy, Pedro, Salomé, y Kiara

Agradecimientos

La finalización de la presente tesis doctoral ha sido el resultado del esfuerzo continuo, de un gran sacrificio realizado a lo largo de varios años de duro trabajo, de la ayuda y participación desinteresada de un gran equipo humano y de sus correspondientes instituciones.

Por todo esto quiero dar mis más sinceros agradecimientos a mis directoras de tesis, las Doctoras: Azucena Gonzalez Coloma, Maria Fe Andres Yeves y Carmen Elisa Díaz Hernández, por haberme aceptado como su estudiante de doctorado, por la confianza que depositaron en mi, por el gran aporte que han hecho en mi formación como futuro investigador y por su increíble asesoría y dedicación en la culminación de esta tesis doctoral.

Tambien quiero agradecer a los Doctores:
Manuel Fraga, por su participación y entera dispocisión en la confirmación estructural de productos propuestos en este trabajo.
Jesús Sanz por sus aportes y enseñanzas en todo lo relacionado con cromaografia gaseosa y espectrometria de masas.
Raimundo Cabrera Pérez y Cristina Giménez Mariño (UDI de Fisiopatología ULL), por su colaboración en la realización de los ensayos de hongos fitopatógenos.
Al Ingeniero Jesús Burillo, por sus grandes esfuerzos en el cultivo, recogida y extracción del material vegetal utilizado en este trabajo.

Agradecer al Consejo Superior de Investigaciones Cientificas (CSIC), instituciones y personal tecnico: Instituto de Ciencias Agrarias (ICA-CSIC), Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), Instituto de Química Orgánica General (IQOG-CSIC).

A la Universidad de Zaragoza y Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) por la colaboración prestada en la elaboración de este trabajo.

A los proyectos de investigación: Produccion biotecnologica de bioplaguicidas: optimizacion y formulacion mediante procesos quimicos y tecnologias de fluidos supercriticos, (CTQ2012-38219-C03-01) ; producción y optimización mediante procesos químicos y biotecnológicos. (CTQ2009-14629-C02-01/PPQ) (Ministerio de Educación y Ciencia, España) y al programa de becas predoctorales JAE-predoc del CSIC. Por la financiación para la elaboración de esta tesis.

A mis padres Guillermo y Sol y a mi tio Esteban por el gran esfuerzo que hicieron para que mi llega a España fuera posible.

Gracias a mis compañeros de trabajo: Paula, Adriana, Patricia, Mariana y Lucia por su gran apoyo de este lado del atlántico. A mis amigos Colombianos en España: Heidy, Malka, AnderSSon, Renso, Jhon, Isaura por su amistad y por hacerme sentir como en casa.

Quiero dar mis más sinceros agradecimientos a Ruben y a Felipe por su amistad, por su apoyo incondicional, por sus palabras de motivación en los malos momentos. Ademas, quiero

agradecerles por todo el apoyo técnico prestado en la realización de esta tesis. A mi familia en España: Angel, Maria Carmen, Lucia, Veronica y Guillermo decirles muchas gracias por hacerme sentir cada dia como uno más de la familia.

Por ultimo, y no por eso menos importante, quiero agradecer a Estefania, mi futura esposa y futura madre de mi hij@, por todo el apoyo que me ha brindado tanto en la parte técnica de mi tesis como en la parte emocional. Muchas gracias cariño por hacerme un hombre feliz.

Presentación:

Este trabajo se ha realizado en el grupo Bioplaguicidas, Biotecnología y Química de Productos Naturales ICA/IPNA (CSIC), y ha sido dirigida por la Dra. Azucena González Coloma, la Dra. Carmen Elisa Díaz Hernández y la Dra. María Fe Andrés Yeves.

El resultado de este trabajo ha dado lugar a las siguientes publicaciones:

Chemical composition and biological effects of essential oils from *Artemisia absinthium* L. cultivated under different environmental conditions. Maria Bailen, Luis F. Julio, Carmen E. Diaz, Jesus Sanz, Rafael A. Martínez-Díaz, Raimundo Cabrera, Jesus Burillo, Azucena Gonzalez-Coloma**.** *Industrial Crops and Products* (**2013**), *49*, 102-107.

Comparative chemistry and insect antifeedant effects of conventional (Clevenger and Soxhlet) and supercritical extracts (CO_2) of two *Lavandula luisieri* populations. Luis F. Julio, Luis Martín, R. Muñoz, Ana M. Mainar, José S.Urieta, Jesus Sanz, Jesús Burillo, and Azucena González-Coloma. *Industrial Crops and Products* (**2014**), 58, 25-30.

Chemical and biocidal characterization of two cultivated *Artemisia absinthium* populations with different domestication levels. Luis F. Julio, Jesús Burillo, Cristina Giménez, Raimundo Cabrera, Carmen E. Díaz, Azucena González-Coloma. *Industrial Crops and Products* (**2015**), *76*, 787-792.

Trypanocidal, trichomonacidal and cytotoxic components of cultivated *Artemisia absinthium* Linnaeus (Asteraceae) essential oil. Rafael A. Martínez-Díaz, Alexandra Ibáñez-Escribano, Jesús Burillo, Lorena de las Heras, Gema del Prado, M. Teresa Agulló-Ortuño, Luis F. Julio, Azucena González-Coloma. *Mem Inst Oswaldo Cruz, Rio de Janeiro* (**2015**), 110, 693-699.

Nematicidal activity of hydrolate from *Artemisia absinthium* var. © candial. Luis F. Julio, Azucena González-Coloma, Jesus Burillo, María Fe Andrés-Yeves. (En elaboración).

Chemical characterization of the nematicidal components of the hydrolate byproduct from *Artemisia absinthium* vapor pressure extraction. Luis F. Julio, Azucena González-Coloma, Jesus Burillo, María Fe Andrés-Yeves, Carmen E. Diaz. (En elaboración).

Phytotoxic and nematicidal components of *Lavandula luisieri.* Luis F. Julio, Alejandro F. Barrero, M. Mar Herrador del Pino, Jesús F. Arteaga, Jesús Burillo, Maria Fe Andres, Carmen E. Díaz and Azucena González-Coloma. (En revisión).

Ixodicidal compounds from cultivated *Lavandula luisieri.* Luis F. Julio, Nadhem Aissani, Felix Valcarcel, Jesus Burillo, Carmen E. Díaz, Sonia Olmeda, Azucena González-Coloma. (En elaboración).

Otros resultados han sido publicados en las siguientes publicaciones y que no aparecen en esta memoria:

Comparative chemistry and insect antifeedant action of traditional (Clevenger and Soxhlet) and supercritical extracts (CO_2) of two cultivated wormwood (Artemisia absinthium L.) populations. Luis Martín, Luis F. Julio, Jesús Burillo, Jesús Sanz, Ana M. Mainar, Azucena González-Coloma. *Industrial Crops and Products* (**2011),** 34, 1615-1621.

Selective nematocidal effects of essential oils from two cultivated *Artemisia absinthium* populations. Juan José García-Rodríguez; María-Fé Andrés; Alexandra Ibañez-Escribano; Luis F Julio; Jesús Burillo; Francisco Bolás-Fernández; Azucena González-Coloma. *Zeitschrift fur Naturforschung* (**2015).** doi: 10.1515/znc-2015-0109.

Chemical composition and phytotoxic effects of aeroponic *Artemisia Absinthium* roots. Luis F. Julio, Azucena González-Coloma, B.M. Fraga, Carmen E. Diaz. (En elaboración).

PATENTE: PRODUCTO BIOCIDA EXTRAIDO DE MATERIAL VEGETAL DE *Lavandula Luiseri*, PROCEDIMIENTO DE OBTENCIÓN Y USO DEL MISMO. Número de solicitud: P201530434 (31 marzo 2015). ES1641.1100. Solicitante: CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS. Autores: A. Gonzalez-Coloma, M. Fe Andres, C.E. Díaz, L.F. Julio, A.F. Barrero, J. Burillo

Otros resultados presentados en los siguientes congresos mediante comunicación oral (*) y/o formato póster.

"*Comparative chemistry and insect antifeedant action of traditional (Clevenger and Soxhlet) and supercritical extracts (CO_2) of two cultivated wormwood (*Artemisia absinthium* L.) populations*". V Mediterranean Organic Chemistry Meeting and III Reunión del Grupo Especializado de Productos Naturales de la RSEQ. **2011.** Cádiz, España.

"Biological and metabolomic profiling of two *Lavandula luisieri* populations in the process of domestication". International Congress "Natural Products and Biocontrol". **2012.** Perpignan, Francia.

"Chemical composition of aeroponic *Artemisia absinthiu*m roots". XXXIV Reunión Bienal de la Real Sociedad Española de Química. **2013.** Santander, España.

"Trypanocidal components of cultivated *Artemisia absinthium* essential oil". XVIII Congreso de la Sociedad Española de Parasitología. **2013.** Gran Canaria, España.

(*) **"Nuevos nematicidas: valorización de residuos de la producción semi/industrial de aceites esenciales".** XVII Congreso de la Sociedad Española de Fitopatología. **2014.** Lleida, España.

"Análisis de los cambios metabólicos inducidos en la interacción tomate/ *Meloidogyne javanica* compatible e incompatible." XVII Congreso de la Sociedad Española de Fitopatología. **2014.** Lleida, España.

El presente trabajo de Tesis Doctoral se ha realizado bajo el marco de los siguientes proyectos de investigación:

"Bioplaguicidas: Producción y Optimización mediante procesos químicos y biotecnológicos". Plan Nacional de I+D+i (2008-2011). Proyecto Coordinado CTQ2009-14629-C02-01 (subprograma PPQ). Ministerio de Ciencia e Innovación.

"Producción Biotecnológica de Bioplaguicidas: Optimización y Formulación mediante procesos químicos y tecnología de fluidos supercríticos". Dirección General de Investigación Científica y técnica, MINECO (CTQ2012-38219-C03-01).

ÍNDICE GENERAL

Resumen

La agricultura y la horticultura se enfrentan a graves problemas de plagas y patógenos. Actualmente, el uso de plaguicidas sintéticos es el método principal de protección empleado. Sin embargo, su uso excesivo ha acarreado el desarrollo de resistencias, contaminación del medio ambiente, toxicidad para organismos beneficiosos y riesgos para la salud humana. Con este trabajo pretendemos contribuir a una protección vegetal más sostenible mediante el desarrollo de bioplaguicidas basado en el uso de extractos de plantas y residuos de su extracción, de aplicación en la UE y en el mercado global. Los extractos se han obtenido a partir de recursos naturales renovables procedentes de la domesticación y cultivo en campo de especies seleccionadas (*Artemisia absinthium* y *Lavandula luisieri*) y los residuos de su extracción. El cribado de bioactividad de los extractos frente a una serie de dianas seleccionadas (insectos plaga, nematodos fitoparásitos, hongos y semillas) se combina con estudios de metabolómica y química para identificar y caracterizar los compuestos bioactivos. Para el estudio de la composición quimica del material vegetal seleccionado hemos usados diferentes partes de la planta que nos permitieron genarar una variedad de extractos entre los que tenemos el aceite esencial y su correspondiente hidrolato, extractos organicos (hexanicos, etanolicos) asi como extracciones con CO_2 supercrítico de las especies seleccionadas. Esta última extracción se considera una alternativa verde a la extracción con disolventes orgánicos así como el enriquecimiento de compuestos bioactivos en los extractos. Las herramientas de producción de biomasa empleadas incluyen la domesticación y cultivo en campo además de cultivo aeropónico.

Abstract

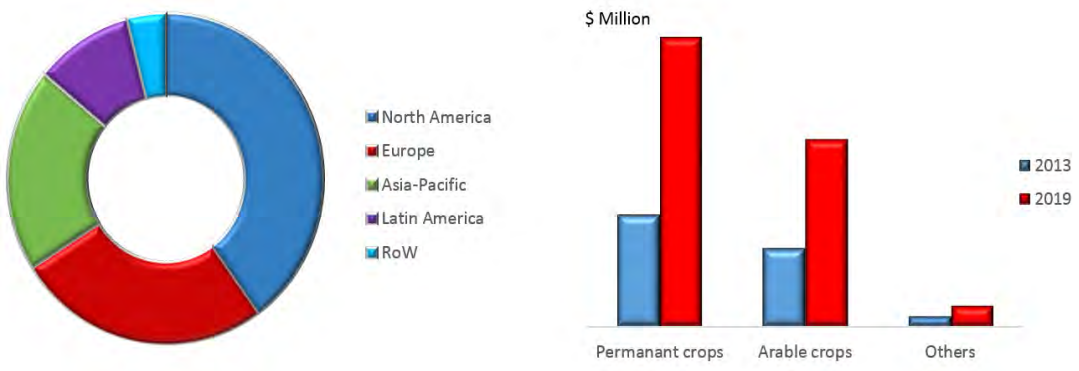
Agriculture and horticulture are facing serious problems of pests and pathogens. Currently, the use of synthetic pesticides is the main method of protection employed. However, their overuse has led to the development of resistance, environmental pollution, toxicity to beneficial organisms and risks to human health. In this work we contribute to a more sustainable crop protection through the development of biopesticides based on the use of plant extracts and waste removal, application in the EU and in the global marketplace. The extracts were obtained from renewable natural resources from the domestication and cultivation of selected species in field (*Artemisia absinthium* and *Lavandula luisieri*) and the waste originated in their extraction. We have combined the screening for bioactivity of the extracts against a number of selected targets (insect pests, plant parasitic nematodes, fungi and plants) combined with chemical and metabolomic studies to identify and characterize the bioactive compounds. To study the chemical composition of the selected plant material we used different parts of the plant that allowed us to generate a variety of extracts including the essential oil and the corresponding hydrolate, organic extracts (hexanic, ethanolic) and CO_2 supercritical. The latter extraction is considered a green alternative to extraction with organic solvents, and allowed for the enrichment of bioactive compounds in the extracts. The biomass production tools employed included field crop domestication and aeroponic cultivation.

1. INTRODUCCIÓN GENERAL

Los bioplaguicidas son productos basados en extractos de materias naturales tales como plantas, bacterias y algunos minerales. Pueden contener microorganismos (bacterias, hongos, virus o protozoos) como ingrediente activo, pueden ser variedades vegetales protegidas genéticamente contra plagas o pueden ser bioquímicos, que incluyen feromonas de insectos y extractos de plantas o microorganismos. Los bioplaguicidas son componentes clave de los programas de control integrado de plagas y enfermedades y están recibiendo mucha atención como medio para reducir los insumos de productos químicos sintéticos.

El mercado mundial de los bioplaguicidas está en expansión con una previsión de € 4.300 millones en 2019, y una tasa de incremento anual del 16,0% (2014-2019). Los factores más importantes de ésta expansión son las regulaciones nuevas y más estrictas relativas a los plaguicidas químicos, la necesidad de cultivos libres de residuos, la expansión de la agricultura ecológica y el aumento de la demanda de productos agrícolas más seguros y respetuosos con el medioambiente (Waterfield and Zilberman, 2012; Popp *et al.*, 2013).

Mercado de bioplaguicidas por zona geográfica, 2013 ($Millones) y tipo de cosecha, 2013-2019



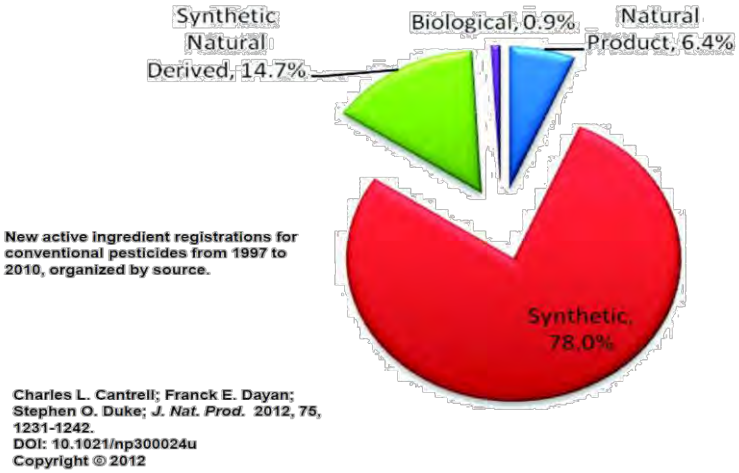
El mercado europeo representa el 45% de la demanda total de bioplaguicidas. La producción hortícola consume el 55% de los bioplaguicidas disponibles, siendo la agricultura ecológica, con un crecimiento anual del 30% en la UE, uno de los consumidores principales. Los bioplaguicidas también se utilizan en salud pública (control vectorial) y silvicultura (*BCC Research Corporation*, http://www.bccresearch.com/report/CHM029B.html).

Muchos plaguicidas sintéticos se han retirado del mercado europeo (Directiva 91/414 CE del Consejo) o se están evaluando (Reglamento 2009/1107 / E y la Directiva 2009/128 / CE). Además, la Directiva 2009/128 / CE del Parlamento Europeo y del Consejo de la Unión Europea establece el marco de actuación comunitaria para el uso sostenible de plaguicidas de los Estados miembros (normativa nacional desde diciembre de 2011), dando prioridad a métodos de alternativos y al Control Integrado de Plagas. A esto hay que añadir el impacto político y social que han tenido los informes sobre los efectos negativos a largo plazo de los insecticidas neonicotinoides en las abejas y su impacto en los cultivos dependientes de polinización (Whitehorn *et al.*, 2012) que han dado lugar a su prohibición en abril de 2013 en la CE (https://sapiens.revues.org/1648). Otro ejemplo es la reciente eliminación del herbicida glifosato en Francia (2015) tras un informe de la OMS sobre posibles efectos cancerígenos.

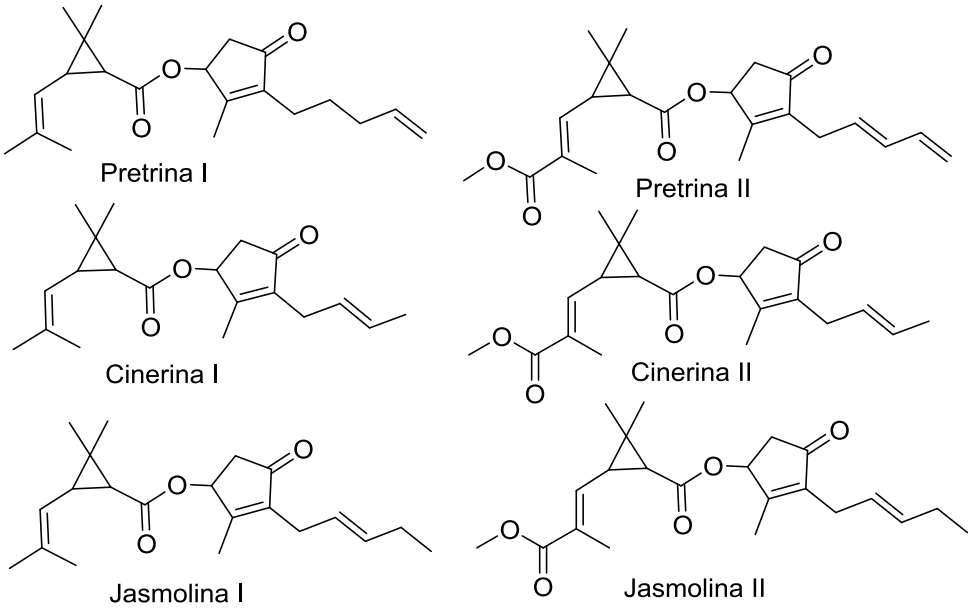
1.1. Origen de los bioplaguicidas basados en productos naturales

La mayoría de los plaguicidas existentes son de origen sintético (78%).

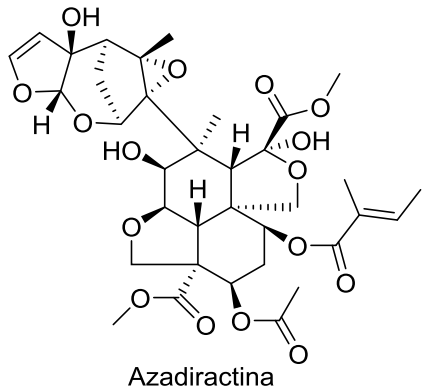
Los plaguicidas basados en productos naturales constan de productos activos obtenidos (puros o en mezclas) de materias primas (botánicos o fúngicos, 6,4 %) o de compuestos sintéticos inspirados en productos naturales bioactivos (con o sin nuevos modos de acción, 15%). Ambos enfoques son complementarios.



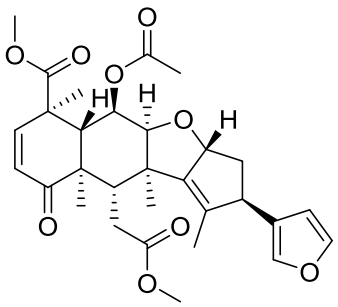
Botánicos: los productos naturales de origen botánico son un segmento importante del mercado de bioplaguicidas, incluyendo productos tales como piretrinas y el aceite de neem para el control de insectos y muchas formulaciones de aceites esenciales. Dentro de las piretrinas se conocen seis sustancias biológicamente activas con propiedades insecticidas. Las moléculas del grupo piretrinas I son ésteres del ácido crisantémico y las del grupo piretrinas II son ésteres del ácido pirétrico.



El aceite de nim es un aceite vegetal extraido de las frutas y semillas del Árbol de nim (*Azadiracta indica*), un árbol de hoja perenne endémico del subcontinente indio y que ha sido introducido en otras muchas zonas de los trópicos. Este aceite es quizás el producto comercialmente más importante de los derivados del árbol de nim. Es usado en agricultura y en medicina. Los componentes activos son azadiractina, nimbina y otros nortriterpenos relacionados.



Azadiractina



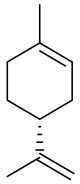
Nimbina

En 1980 menos del 2% de los artículos publicados trataban sobre los insecticidas botánicos, pero han superado el 21% en 2011 (Isman, 2006, 2008; 2014). A diferencia de los plaguicidas convencionales que se basan en un solo ingrediente, los biocidas botánicos incluyen múltiples compuestos que disminuyen el riesgo de desarrollo de resistencias por parte de las plagas (González-Coloma *et al*., 2010).

Agroresiduos: miles de millones de toneladas métricas de biomasa se generan cada año por la industria agrícola en todo el mundo. Entre el 10 y el 60% de los residuos de producción agroindustriales se desperdician anualmente. En este sentido, cada año se generan más de 250 TM de residuos de la industria agroalimentaria según FAOSTAT (2012). Estos residuos orgánicos se consideran sustancias sin valor que requieren una gestión según la normativa vigente, (Directiva 2008/98 / CE relativa a los residuos). Por tanto, un uso eficiente de los residuos agrícolas para la producción de recursos de valor añadido es atractivo y socialmente beneficioso (Vani and Doble, 2011; Santana-Méridas *et al.*, 2012). Las cáscaras de cítricos, incluida la bergamota, se están utilizando en la obtención de aceites esenciales ricos en limoneno para el control de mosquitos (Di Donna *et al.*, 2011). Otra fuente importante de productos químicos son los residuos de la extracción de aceites esenciales (Navarrete *et al.*, 2011) como los residuos sólidos de la extracción de hoja de laurel se emplean para la producción de antioxidantes, terpenos y fenoles (Di Leo *et al.*, 2009), o ácido ascórbico y compuestos anticolesterolémicos del hidrosol del aceite de bergamota (Pernice *et al.*, 2009; Di Donna *et al.*, 2011). Por lo tanto, los residuos agrícolas y desechos de producción de aceites esenciales pueden ser fuentes importantes, renovables y baratas de compuestos valiosos, sin embargo, poco se sabe sobre su uso como fuente de protectores de cultivos naturales (Santana-Méridas *et al.*, 2012).

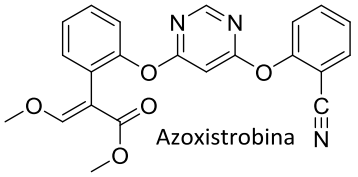
Bergamota recolectada

Aceite esencial de bergamota

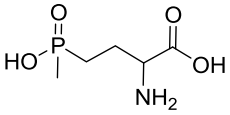


Limoneno

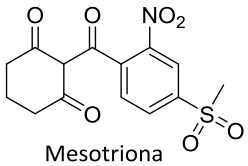
Nuevos productos basados en sustancias naturales: Los productos naturales bioactivos pueden proporcionar el andamio molecular para los derivados sintéticos. El impacto de algunos de los plaguicidas inspirados en productos naturales (no bioplaguicidas) ha sido enorme. Fungicidas a base de estrobilurinas (por ejemplo, azoxistrobina), versiones sintéticas (glufosinato, mesotriona) de los herbicidas naturales fosfinotricina y leptospermona (Duke and Dayan, 2013; Seiber *et al.*, 2014). Insecticidas inspirados en los piretroides (p.e. permetrina) así como los neonicotinoides (imidacloprid) basados en la nicotina y los que actúan sobre receptores de rianodina (flubendiamida). También las espinosinas, obtenidas de la fermentación de dos especies de *Saccharopolyspora,* muestran actividad selectiva contra insectos y se han utilizado como cabezas de serie de insecticidas semisintéticos (Kirst, 2010).



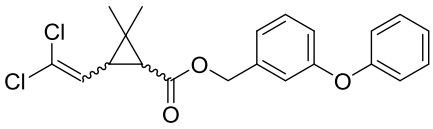
Azoxistrobina



Glufosinato



Mesotriona



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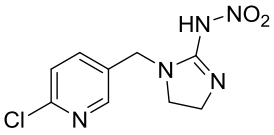

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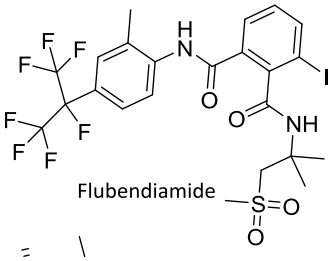

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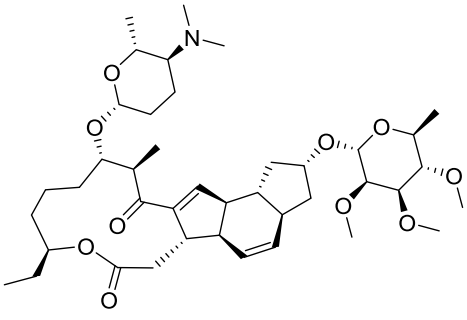
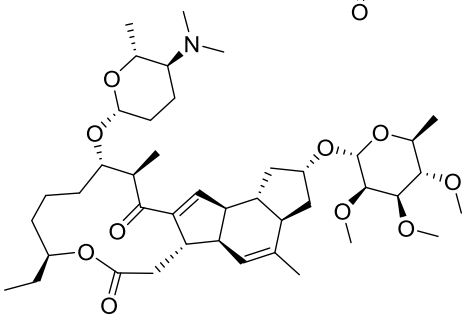


Espinosina A



Espinosina D

1.2. Necesidades y oportunidades

Disponibilidad: Los bioplaguicidas botánicos tienden a ser más caros que sus equivalentes sintéticos y algunos no son producidos en cantidades suficientes o no están disponibles en el mercado (por ejemplo, nicotina). Por lo tanto, el desarrollo de un producto reproducible y sostenible como una clara alternativa a la síntesis orgánica a gran escala puede incluir: Cultivo

aeropónico y cultivo *in vitro* de raíces transformadas en ambientes controlados (Hayden, 2006; Srivastava and Srivastava, 2007). Estos sistemas proporcionan una fuente sostenible de metabolitos (Xu *et al.*, 2011). Sin embargo, no todas las especies se pueden cultivar en estos sistemas artificiales. La domesticación y el cultivo en campo de especies seleccionadas es el enfoque más económico para la producción de biomasa (Prakasa Rao, 2009). La aplicación de buenas prácticas agrícolas y el establecimiento de protocolos de control de calidad de procesos son necesarios para garantizar la calidad del producto final.

Producción limpia y optimización: El dióxido de carbono (CO_2) es inerte, barato y fácilmente reutilizable por lo que se considera un disolvente verde y su uso contribuye a la reducción de emisiones de CO_2 que es la causa principal del calentamiento global. La extracción con CO_2 supercrítico (SCE) es la aplicación más conocida. La SCE se ha aplicado a una amplia variedad de materiales, siendo las plantas uno de los más frecuentes. Las técnicas de extracción tradicionales (orgánica en Soxhlet o hidrodestilación) tienen serios inconvenientes ya que los disolventes orgánicos son volátiles y la hidrodestilación produce descomposición térmica o hidrólisis de compuestos. En este sentido, las tecnologías de extracción con CO_2 supercrícitco (SCE) proporcionan metodologías limpias y sostenibles. Por otra parte, la capacidad de los fluidos SC para extraer diferentes grupos de compuestos con diferentes condiciones se puede aplicar para obtener extractos con actividades específicas que ofrecen un uso completo de las materias primas (González-Coloma et al., 2012; Keplan *et al.*, 2002; Martín et al., 2012).

Nuevos bionematicidas. Esto se debe a la eliminación (Protocolo de Montreal) del bromuro de metilo y otros fumigantes como el isotiocianato de metilo (MITC) y cloropicrina. Los bionematicidas basados en productos naturales son prometedoras (Chitwood, 2002; Ntalli et al, 2011; Andrés et al, 2012).

Herbicidas. Debido a la rápida evolución y propagación de la resistencia a los herbicidas sintéticos por la adopción generalizada de cultivos transgénicos resistentes al glifosato. Existen fitotoxinas naturales que podrían considerarse bioherbicidas y que tienen nuevos modos de acción (Duke y Dayan, 2013).

Otros. Atrayentes y repelentes de plagas de artrópodos (Borges et al, 2011), incluyendo acaricidas-ixodicidas y vectores de parásitos humanos (Paluch et al, 2011; Sainz et al, 2012) y fungicidas naturales (Romanazzi, et al 2012).

2. OBJETIVOS

El objetivo general de esta propuesta de investigación es la producción sostenible de nuevos bioplaguicidas botánicos a partir de las especies *Artemisia absinthium* y *Lavandula luisieri*, seleccionadas en base a sus antecedentes quimiotaxonómicos y la disponibilidad de sistemas de producción sostenible: adaptación y domesticación a cultivo de campo y producción en cultivos artificiales. Se ha estudiado la acción biocida de sus extractos (aceites esenciales y extractos etanólicos) y residuos de la extracción de los aceites esenciales (hidrolatos), los perfiles químicos y los componentes activos. Además se han optimizado sus extractos mediante extracciones con CO_2 supercrítico.

Objetivos especificos

1 Valorización de extractos de Artemisia absinthium en fase final de domesticación.

- Caracterización química y biológica del aceite esencial semi-industrial.
- Caracterización química y biológica del hidrolato.

2. Valorización de extractos de Lavandula luisieri en fase inicial de domesticación.

- Estudio químico y biológico del aceite esencial, hidrolato y extractos orgánicos.

3. Optimización de los extractos mediante extracción supercrítica con CO2.

3. MATERIALES Y METODOS

3.1. Especies vegetales seleccionadas:

3.1.1. *Artemisia absinthium* L.

Reino:	Plantae	Familia:	Asteraceae
Subreino:	Tracheobionta	Subfamilia:	Asteroideae
División:	Magnoliophyta	Tribu:	Anthemideae
Clase:	Magnoliopsida	Subtribu:	Artemisiinae
Subclase:	Asteridae	Género:	*Artemisia*
Orden:	Asterales	Especie:	*A. absinthium*

Es una planta perenne, con la cepa leñosa y ramas herbáceas que pueden alcanzar más de un metro de altura. El tallo es leñoso es asurcado y con numerosas hojas alternas. Las hojas son de un color gris blanquecino en el envés y verde claro en el haz, las hojas inferiores del tallo son muy pecioladas, mientras que las superiores son casi sentadas y divididas en lóbulos. Las flores son tubulosas, amarillentas y están reunidas formando cabezuelas mirando hacia el suelo, las brácteas son de color verde blanquecino. Los frutos son aquenios lisos con forma ovalada (Burillo, 2009).

Se puede encontrar en casi toda Europa y el Magreb occidental, ya que originalmente se distribuyó desde Europa Occidental hasta Asia Central y en la actualidad es posible encontrarla en casi todo el mundo. En España se cría en toda la mitad septentrional de la península, y más puntualmente en las provincias de Valencia, Murcia y Granada (Sierra Nevada).

3.1.2. *Lavandula luisieri* L.

Reino:	Plantae	Familia:	Lamiaceae
Subreino:	Tracheobionta	Subfamilia:	Nepetoideae
División:	Spermatophyta	Tribu:	Lavanduleae
Clase:	Magnoliopsida	Género:	*Lavandula*
Subclase:	Asteridae	Especie:	*L. stoechas luisieri*
Orden:	Lamiales		

Es una mata que puede alcanzar 1 m de altura, muy ramificada. Sus ramas son de color verde o rojizo, blanquecinas por la presencia de pilosidad, más o menos abundante. De follaje gris verdoso. Tiene una inflorescencia bastante gruesa y alargada que lleva en su terminación un conjunto de brácteas estériles de color violeta o rojizo. El cáliz es pequeño y queda oculto por la bráctea, es recorrido por 13 venas. Corola de color morado oscuro. Las flores forman líneas verticales que recorren el conjunto de la inflorescencia. El pedúnculo corto o mediano, el cáliz con pelos todos muy cortos, y la corola con todos los lóbulos iguales o poco desiguales. Esporofilos de 4-8 mm de largo, acorazonados-romboidales, de color violeta amarronado, membranosos, tomentosos de gris en 4 hileras, los superiores agrandados, sin flores axilares, de 1-5 cm de largo, ovalados, púrpuras hasta violeta azulado. El penacho coloreado sirve para atraer a los insectos.

Está repartida por el centro y la mitad occidental de la Península Ibérica.

3.2. Cultivo aeropónico

Se utilizaron plántulas de *Artemisia absinthium* procedentes de semilleros y cultivadas en una cámara de cultivo a 25 ºC, 70% RH, 16:8 fotoperíodo. Las plántulas se transfirieron a una cámara de cultivo aeropónico (Figura 1). La cámara aeropónica tiene unas dimensiones de 4 x 1.5 x 0.4 metros y un tanque de agua de 220 L. Las plantas se colocaron sobre un soporte inerte de arcilla expandida manteniendo una separación entre ellas de 20 cm y se cultivaron a 20-25 ºC con luz artificial bajo un régimen de fotoperiodo de 16 h luz: 8 h oscuridad. Se pulverizaron las raíces 15 minutos cada 2 h con una solución nutritiva 0.2 g/L de Nutrichem (20:20:20 de N, P, K -Miller Chemical & Fertilizer Corp) y 0.03 % H_2O_2 (33% w/v Panreac). Partes aéreas y raíces se recolectaron cuando su longitud alcanzaba los 20-30 cm para obtener los correspondientes extractos.

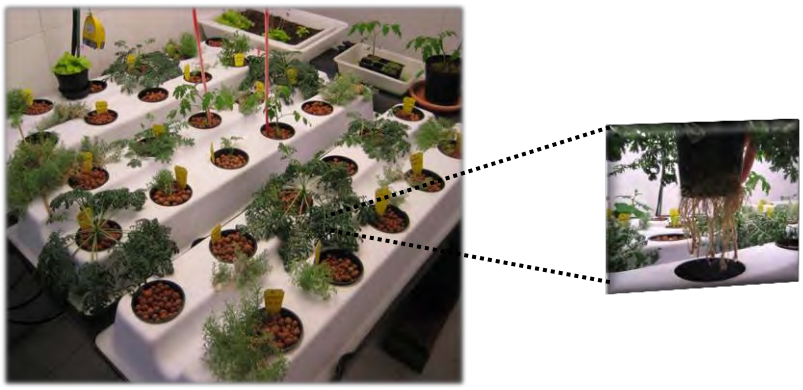


Figura 1. Cámara de cultivo aeropónico

3.2. Organismos diana:

3.2.1. Insectos

3.2.1.1. ***Spodoptera littoralis*** (Bois) (Balachowsky, 1972), conocida también como rosquilla negra.

Esta especie es muy polífaga y se conoce por su extrema voracidad. Los principales cultivos afectados son los hortícolas, pimiento, alfalfa, maíz, algodón, tomate, patata entre otros. La oruga es de color negruzco y se arrolla en espiral, las orugas tienen actividad nocturna y comen cualquier parte verde de la planta y los frutos. Presenta seis estadías larvales y en su mayor desarrollo puede alcanzar de 3.5 a 4 cm de longitud. Los adultos se caracterizan por llevar dibujado, en las alas anteriores, un 4 en la derecha y su simétrico en la izquierda. Las larvas se alimentan tanto de hojas, flores y frutos, llegando a alcanzar un marcado gregarismo. Esto unido a su alta capacidad reproductora y de migración les confiere una gran importancia económica en el Norte de África y Europa meridional.

3.2.1.2. *Myzus persicae* (Sulzer). Pulgón verde del melocotonero.

Es un pulgón de tamaño medio, de 1.2 a 2.3 mm. Las formas ápteras presentan color verde amarillento y entre verde y negro las formas aladas. El hospedero primario suele ser una planta del género *Prunus*, sobre todo *Prunus persica* (L.), aunque es muy polífago y presenta más de cuarenta familias de hospedadores secundarios, muchas de ellas de elevado interés económico. Es capaz de transmitir más de 100 tipos de virus, (Kennedy and Day 1962) entre los que encontramos algunos muy persistentes como luteovirus del amarilleo occidental de la remolacha, el penamovirus de las excrecencias y mosaico del guisante, o el luteovirus del enrollado de la patata, virus semipersistentes como el closterovirus del amarilleo de la remolacha o el caulimovirus del mosaico de la coliflor, y no persistentes como el virus del mosaico de la alfalfa, el potyvirus latente de la alcachofa, el potyvirus del mosaico amarillo de la judia, el fabavirus del marchitamiento del haba, el potyvirus del mosaico de la lechuga y de la remolacha. Al igual que su hospedador primario, es originario de Asia aunque actualmente se considera cosmopolita (Blackman R.L. & Eastop V.T. 1984).

3.2.1.3. *Rhopalosiphum padi* (L). Pulgón de la avena, pulgón de los cereales.

Es uno de los 14 áfidos considerados dentro de los de mayor importancia económica en el mundo y es altamente polífaga. Su tamaño varía entre 1.2 y 2.4 mm, su color va desde el verde al negro grisáceo, aunque generalmente es de color verde oliva; igual que en el caso de *Myzus persicae,* el hospedador primario suele ser una planta del género *Prunus*, generalmente *Prunus padus* (L.). En cuanto a su hospedador secundario es bastante polífago. Prefiere las gramíneas, incluidos los cereales y las plantas de pasto. Es vector de virus vegetales persistentes y algunos no persistentes: BYDV (virus de la hoja manchada del maíz), el potyvirus del enanismo amarillo de la cebolla, potyvirus del mosaico enanizante del maíz. Se distribuye por todo el mundo (Blackman R.L. & Eastop V.T. 1984).

Las poblaciones de *S. littoralis*, *L. decemlineata* y pulgones se mantuvieron en cámaras climatizadas a una temperatura de 22 ± 1 ºC, humedad relativa del 60-70% y fotoperíodo de 16:8 horas (luz:oscuridad). *S. littoralis* se cría en cajas de plástico de diferentes dimensiones dependiendo de la estadía larval, tamaño y número. Las larvas se alimentaron con una dieta artificial general para noctúidos (Poitout S. and Bues S., 1970) y los adultos con solución azucarada. En el caso de *L. decemlineata* las poblaciones se mantuvieron en cajas de plástico ventiladas, alimentándose de partes aéreas de plantas de papa (*Solanum tuberosum* L. cv Desirée) cultivadas en invernadero. Los pulgones se crían en cámaras climatizadas sobre sus hospedadores. *M. persicae* sobre plantas de pimiento (*Capsicum annuum* L. var. Califronia Wonder) y *R. padi* sobre cebada (*Hordeum vulgare*), en jaulones ventilados, transfiriéndoles a plantas frescas cada 7-10 días.

3.2.2. Garrapatas

3.2.2.1. *Hyalomma lusitanicum*.

Las garrapatas del género *Hyalomma* (unas 30 especies) son muy abundantes en zonas áridas de vastas regiones de Asia, Europa mediterránea y África. Según las especies atacan a todo tipo de ganado (bovinos, ovinos, caprinos, etc.) y otros mamíferos salvajes, aves y reptiles, a perros y gatos, y también al hombre. Las garrapatas del género *Hyalomma* son de talla media a grande, con piezas bucales prominentes. La mayoría de las especies son de 3 hospedadores, pero también las hay de 1 y 2 hospedadores. Una singularidad de este género es que algunas especies pueden utilizar de uno a tres hospedadores para desarrollarse. El ciclo vital puede durar entre 3-4 meses y más de un año, según la especie y el clima. Las ninfas y los adultos pasan el invierno en grietas y rendijas entre las piedras de muros y establos, o en praderas no cultivadas. *H. lusitanicum* es de distribución estrictamente mediterránea (Estrada-Peña et al. 2004). En España es la especie de garrapata más abundante en el Centro de la Península Ibérica (86%) (Barandika *et al.*, 2011).

Las hembras grávidas de *H. lusitanicum* son capturadas regularmente en la finca "La Garganta", localizada en el sur de la provincia de Ciudad Real a 37º 24' 78'' N 42º 59' 101'' E a 669 m.s.n.m., y se colocan en tubos de ensayo de vidrio con un trozo de papel de filtro cortado en zig-zag en el interior y un tapón de algodón. Los tubos se incuban a 24 ºC con una humedad relativa superior al 70% hasta que tiene lugar la oviposición. Los huevos se dejan alrededor de 23-24 hasta la eclosión. Después, las larvas de *H. lusitanicum* necesitan 15-16 días para completar la quitinización (Ouhelli and Pandey, 1984).

3.2.3. Nematodos fitoparásitos

3.2.3.1. *Meloidogyne javanica*

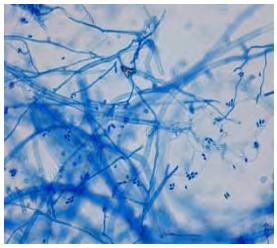
Los nematodos formadores de nódulos del género *Meloidogyne* son uno de los principales patógenos de especies vegetales y presentan una distribución mundial. Son endoparásitos obligados, de naturaleza polifaga e infectan más de 3000 plantas cultivadas a las que causan severos daños con las consiguientes pérdidas económicas. La infección se produce cuando el juvenil de segunda edad, móvil e infectivo, es atraído por el sistema radical de la planta huesped. Despues de penetrar por el apice de la raíz, migra intercelularmente hasta las proximidades del cilindro vascular donde seleccionna un grupo de células vegetales que se transforman en su sitio de alimentación. Durante todo su cilo vital el nematodo permanece fijado en el sitio de alimentación a traves del cual extraen de la planta huesped agua y nutrientes. La infeccion afecta al crecimiento de la planta, causa marchitamiento, aumenta su susceptibilidad a otros patógenos y en determinadas condiciones puede causar su muerte.

El mantenimiento de las poblaciones de nematodos se realiza en plantas de tomate (var Marmande). Los semilleros se preparan en yiffis con vermiculita y se mantienen en

cámara a 24 º C. Aproximadamente unos 25 días después de la germinación se trasplantan las plántulas en su ubicación definitiva (maceta de 12 cm de diámetro) con sustrato de suelo areno limoso, de forma individualizada se procede a la inoculación (bien directamente con juveniles J2 o colocando un trozo de raíz infectada). Pasados dos meses de la inoculación se levantan las plantas, se lavan las raíces y se extraen manualmente las masas de huevos bajo microscopio estereoscópico. Dichas masas se colocan con la ayuda de pinzas en filtros semisumergidos en un pequeño recipiente con agua destilada.Los filtros se incuban en la cámara a 24ºC para que los huevos comiencen a eclosionar. Aproximadamente durante un mes iremos obteniendo juveniles J2 que constituiran el inóculo posteriormente utilizaremos en las concentraciones adecuadas para los ensayos de actividad.

3.2.4. Hongos fitopatógenos

3.2.4.1 *Fusarium* sp.



El género *Fusarium* pertenece al grupo de los Deuteromicetes, relacionándose las formas asexuales de *Fusarium solani* con las formas Ascomicetas. Es responsable de muchas enfermedades en los vegetales, como son: marchitamiento, podredumbres radiculares y de tallos, podredumbre de frutos en general, alteraciones de la madera, etc. Además, produce la podredumbre seca de los tubérculos de la papa y causa necrosis en raíces de tomate y berenjena.

El mantenimiento de las colonias se realizó en medio PDA comercial (Oxoid) en placas petri. A la hora de preparar el medio de cultivo se le añadió el antibiótico cloranfenicol (50 mg/l) para evitar la proliferación de microorganismos. Las placas se mantuvieron en la oscuridad y en una estufa, a 27 ºC. De manera periódica se realizaron resiembras para mantener las colonias en condiciones óptimas. Al mismo tiempo se tomaron tacos de agar de las diferentes placas y se transfirieron a eppendorf con glicerol al 18% para su conservación a -80ºC.

3.3. SEMILLAS

Se utilizaron especies vegetales de *Lolium perenne* L. (una hierba invasiva que compite con los cultivos) y *Lactuca sativa*, semilla empleada habitualmente en los estudios de interacciones alelopáticas. Las especies de *L. perenne* y *L. sativa* fueron seleccionados por su capacidad germinativa y por su crecimiento rápido (<72h y <24h respectivamente).

3.4. BIOENSAYOS DE ACTIVIDAD

3.4.1. Actividad antialimentaria (insectos-plagas)

S. littoralis y *L. decemlineata:* Se basa en la preferencia de larvas del sexto estadío (L6) de *S. littoralis* y/o adultos de *L. decemlineata* por discos de hojas (tratados y control) de la planta huésped de *C. annuum* y *S. tuberosum*, respectivamente, colocados en placas Petri.

Los extractos crudos se ensayaron a una dosis inicial de 100 µg/cm^2. En el caso de los productos puros se ensayaron a una dosis inicial de 50 µg/cm^2. La superficie de los discos (1

cm^2) se trató superficialmente con una solución stock de extracto o producto. Cada ensayo consistió en 6 placas petri con dos y tres insectos de *S. littoralis* y *L. decemlineata* por placa, respectivamente. Una vez consumido el 75% de la superficie de los discos control o tratamiento, las áreas foliares restantes se midieron (http://imagej.nih.gov/ij) y se calculó el índice de consumo (%FR) (Bentley et al., 1984) mediante la ecuación: %FR = 1- (consumo de tratamiento/consumo del control) x 100. Figura 2 A

Con *M. persicae* se emplearon dos medios discos de hojas de *C. annuum*, mientras que con *R. padi* se utilizaron dos fragmentos de hoja de *H. vulgare* (1 cm^2 cada uno) por caja. Cada fragmento de hoja se trató con el extracto o producto a ensayar (tratamiento) y la otra mitad o fragmento se trató con el disolvente (control). Las cajas se incubaron a 22 ± 1 ºC y 16:8 horas (luz:oscuridad) colocadas en posición invertida y bajo luz indirecta durante 24 horas. Después de 24 horas se contaron los pulgones asentados en el tratamiento y en el control, calculándose el índice de inhibición del asentamiento (%SI) para cada tratamiento: (Gutiérrez et al., 1997) %SI = 1- (%T/%C) x 100, dónde %T es el porcentaje de pulgones asentados sobre la superficie tratada y %C es el porcentaje de pulgones asentados sobre el control. Se realizan estudios de dosis-respuesta cuando el FR / SI >75%. Figura 2 B

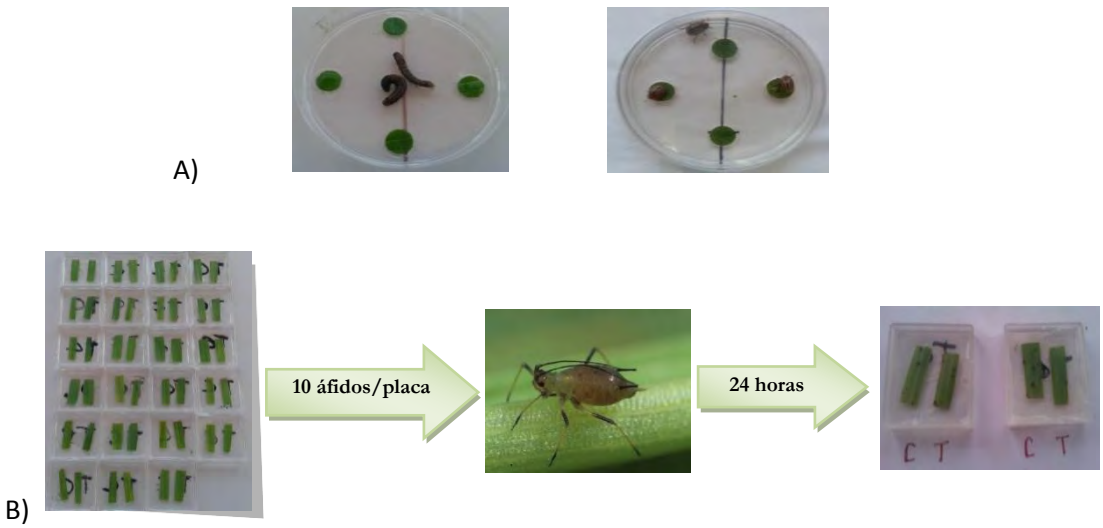


Figura 2. Bioensayos de elección : A) *S. littoralis* en pimiento B) *R. padi* en cebada.

3.4.2. Actividad ixodicida.

3.4.2.1. Toxicidad

Para los ensayos se utilizaron 20 larvas de *H. lusitanicum* de 10 días de vida o 10 adultos por repetición, con la finalidad de determinar la actividad ixodicida (González-Coloma, A et al., 2012) de los extractos y productos. Tubos Eppendorf con 25 / 150 mg de celulosa (Celulosa cristalizada Merk) se trataron con 50 / 300 µl de una solución stock (20 y 10 mg/ml, de extracto o producto) y se deja evaporar el disolvente. Como control positivo se utiliza acibelte (10 µg/µl), y disolvente (acetona o metanol según corresponda) como control negativo, el ensayo se realiza por triplicado. El contenido de los eppendorf se aplica sobre los lotes de larvas o adultos de garrapatas en los tubos de ensayo, se tapan con algodón hidrófilo, se homogeniza la mezcla y se mantienen a 24 ºC/HR>70% durante 24 horas (Figura 3). Una vez

finalizado el experimento se contabiliza la mortalidad de las larvas /adultos de *H. lusitanicum* y se corrige respecto a la mortalidad del control negativo de acuerdo con la ecuación de Schneider Orelli: % M= (%T-%C/100-%C) x 100, %T es el porcentaje de garrapatas muertas del tratamiento y %C el porcentaje de garrapatas del control negativo (disolvente).

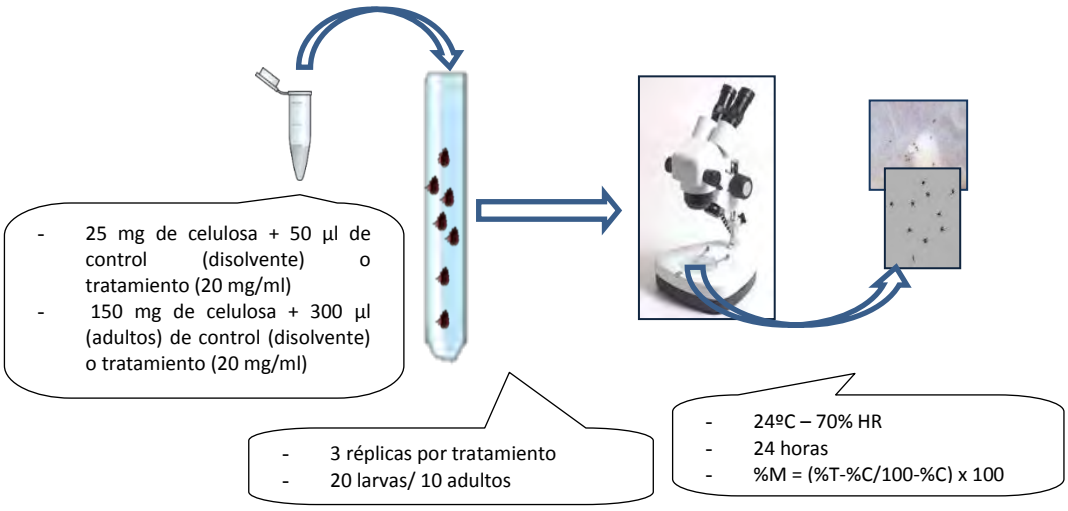


Figura 3. Esquema experimental de los bioensayos con *H. lusitanicum*

3.4.3. Actividad nematicida

3.4.3.1. Ensayos *in vitro* : mortalidad de J2

Se llevan a cabo en placas de plástico de 96 pocillos (con fondo en U) con 4 réplicas para cada tratamiento, además del control (Figura 4). Los pocillos de alrededor se rellenan con agua para evitar la desecación. En el caso de los extractos acuosos (hidrolato) la solución con el inóculo se pasa por un filtro para que queden retenidos en él los nemátodos, y seguidamente se lavan y arrastran con el volumen (100 µl) de tratamiento que se va a ensayar, o con el mismo volumen de agua en el caso del control rellenándose los pocillos, convenientemente marcados para su identificación. En el caso del aceite esencial, a cada pocillo se añaden 95 µl de la solución de agua con nematodos más 5 µl del aceite a una concentración de 20 µl, para de este modo obtener una concentración final del tratamiento de 1µg/µl. Asi mismo los controles incluirán 95 µl de la solución con nematodos más 5µl del disolvente utilizado (DMSO+Tween 0.6%). Figura 4.

La distribución de las muestras se realizará en grupos de 4 al azar y dejando la fila y la columna externas rellenas de agua para mantener la humedad y evitar el efecto borde. Posteriormente se procede a sellar dicha placa con parafilm y envolverla con papel plata que impida el paso de luz. Se incuba a 24ºC durante 72 horas, y si muestran actividad se ensayan también a tiempos menores (24 y 48 horas). Pasado el periodo de incubación se realiza el recuento de nematodos muertos y vivos en un microscopio estereoscópico. Los datos de actividad nematicida se presentan como porcentaje J2 muertos corregido según la fórmula de Scheider-Orelli. Las dosis letales eficaces (LC_{50} y LC_{90}) se calcularon mediante análisis Probit con los resultados obtenidos en distintas concentraciones, empleando para ello el programa estadístico Statgraphics Centurion.

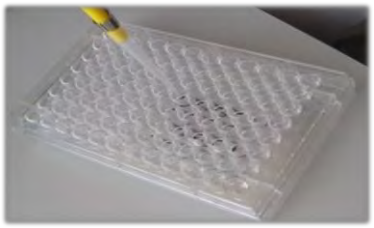


Figura 4. Placa estéril de 96 pocillos con fondo en U para la realización de ensayos de actividad con nematodos infectivos J2.

3.4.3.2. Ensayos in vitro : Efecto sobre eclosión de masas de huevo

Se colocan 3 masas de huevos (obtenidas manualmente de raíces infectadas tal y como se detalla en el apartado anterior) para cada réplica en un filtro con un tamaño de poro que permite el paso de los nemátodos J2 que eclosionan de las masas, pero no permite el paso de éstas. Estos filtros se colocan a su vez en una placa de plástico de 24 pocillos grandes, con fondo plano y 400 µl del tratamiento o del control (agua). Se incluyen cuatro repeticiones de tratamiento y cuatro de control. La placa se cubre para evitar la evaporación y se incuba en oscuridad a 25ºC Figura 5. Pasado este tiempo se realiza el primer recuento de juveniles eclosionados y se procede a retirar la solución con tratamiento y a sustituirla por agua destilada estéril. La eclosión de los huevos se monitoriza durante 4 semanas, una vez por semana.

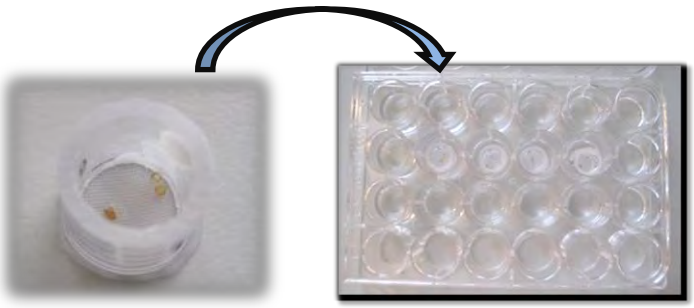


Figura 5. Filtro que contiene las masas de huevos que serán colocadas en la placa de 24 pocillos con los respectivos tratamientos.

3.4.3.3. Ensayos In vivo: efectos en la capacidad de infección de nematodos (J2).

En estos ensayos se evalúa la actividad del extracto sobre la capacidad infectiva en raíces de tomate. Para ello, se realiza un ensayo con nemátodos J2 en placa de 96 pocillos tal y como se especifica en el apartado de ensayos de actividad, pero en este caso con una dosis de tratamiento no-letal. Tras 72 horas de incubación, el inóculo se filtra, se lava y se aplica a las plántulas de tomates que previamente han sido trasplantados en pequeñas macetas con arena estéril. Se realizan 6 réplicas con el tratamiento, con sus respectivos controles inoculados con J2 sin tratar. Los tomates se mantienen en cámara (25 ± 2 °C, 60% HR, 16:8 D:N) durante una semana, tras la cual se procede al procesamiento de las raíces y su tinción. Estas se lavan con una solución de lejía, y posteriormente con agua, y se colocan

separadamente en vasos de precipitados pequeños a los que se añaden 40 ml de agua y 1 ml de colorante fucsina. Se llevan a ebullición en un microondas y se dejan enfriar, se lavan las raíces y se conservan en frío y tapadas durante 24 h. Una vez transcurrido ese tiempo, se cuantifica número de J2 en el interior de las raíces bajo con un microscopio estereoscópico (Figura 6).

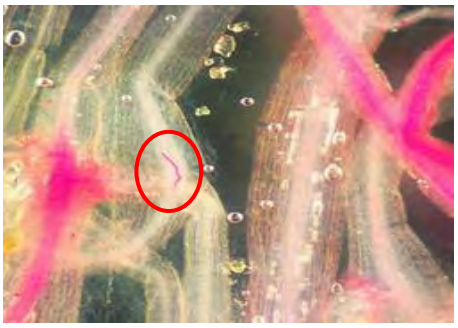


Figura 6. Imagen de una raíz de tomate infectada en un ensayo de penetración vista al microscopio tras la tinción. En el círculo central se observa un J2 que ha penetrado en la raíz**.**

3.4.3.4. Ensayos In vivo: efectos en la reproducción de la población de *M. javanica* en plantas de tomate.

Para los experimentos en maceta se mezclan 10 ml de la solución tratamiento con 1.200 Kg de suelo humedecido de textura areno-limosa que se transfiere a una maceta de plástico de 1L de capacidad. Cada maceta se inocula con 2000 juveniles de *M. javanica* y se incuban durante 5 dias en una cámara de crecimiento vegetal (25 ± 2 °C, 60% HR, 16:8 D:N). Como controles se utiliza suelo no tratado. Despues del periodo de incubación, se trasplanta a cada maceta una plántula de tomate de 1 mes de edad. Cada tratmiento tiene cinco réplicas y se mantienen en la cámara de crecimiento vegetal durante dos meses. Al final del experimento se procesa el sistema radical según la metodología de Verdejo Lucas et al (2012), que permite cuantificar el número de masas de huevos y de juveniles/huevos por planta. La infectividad de los nematodod se estima a partir de la cuantificación del número de masas de huevos por planta. Los resultados obtenidos se transforman en [log10 (x+1)] para su análisis estadístico mediante ANOVA y LSD ($P<0,05$).

3.4.4. Actividad antifúngica

La actividad fungicida de extractos y compuestos, se realizó mediante dilución en agar en placas Petri. Se emplearon 3 concentraciones diferentes (1, 0.5 y 0.1 mg/ml). Los extractos o productos se incorporaron disueltos en etanol (2%) al medio de cultivo. Se sembraron 8 réplicas de *F. solani* por muestra y se incubó a 27 ºC durante 72 horas (Figura 7). Transcurrido ese tiempo se digitalizaron las imágenes y se midieron las colonias (http://imagej.nih.gov/ij) para calcular el % de inhibición del crecimiento y calcular las dosis efectivas EC_{50} (STATGRAPHICS centurium XVI). El porcentaje de inhibición (%I) del crecimiento se calcula según la fórmula %I= [(control-Tratamiento)/Control] x 100.

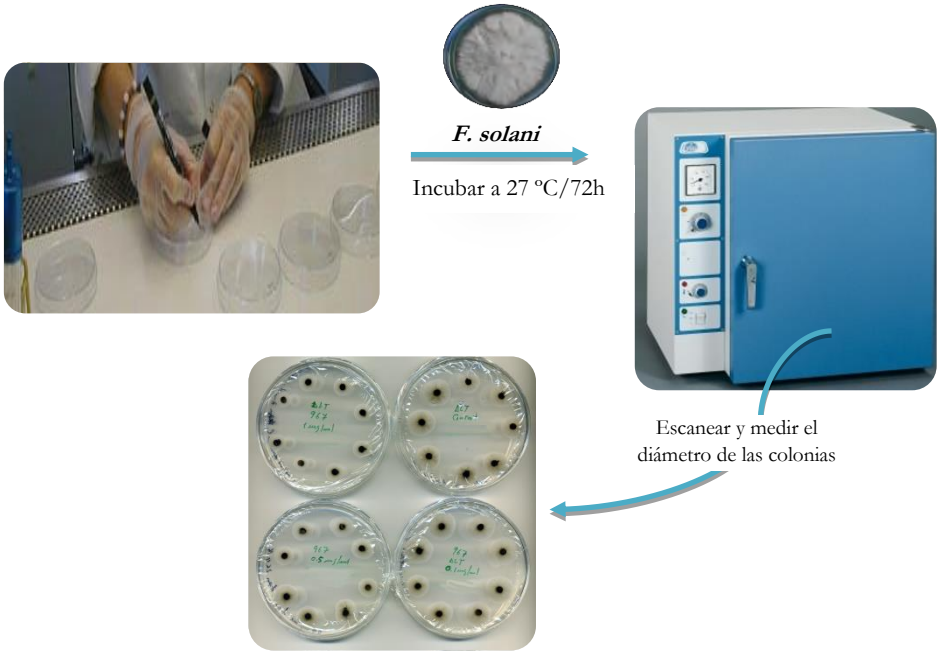


Figura 7. Ensayo de actividad antifúngica frente a *F. solani*.

3.4.5. Actividad fitotóxica.

Se estudió el efecto de los extractos y productos puros sobre la germinación de las semillas, longitud radicular y foliar de *L. perenne* y sobre la germinación y longitud radicular de *L. sativa.*

El ensayo se realizó en placas de 12 pocillos (2 cm^2 cada pozo). En cada pocillo se incluyeron discos de papel (Whatman nº 1, 2.0 cm de diámetro) tratados con 20 µl de extracto o compuesto (100 y 50 µg/cm^2), disolvente como control, 10 semillas y 500 µl de agua. Las pacas se taparon, sellaron con parafilm y se colocaron en una cámara de cultivo vegetal (25 ± 1 °C, HR>70%, L:O 16:8 h) durante 7 días. Se realizan 4 réplicas por muestra. La germinación se controló cada 24 h hasta completar los 7 días, empezando a las 72 horas para *L. perenne* y a las 24 horas para *L. sativa* (Figura 8). Al final del experimento se midió la longitud radicular (*L. sativa* y *L. perenne*) y foliar (*L. perenne*) digitalmente (http://imagej.nih.gov/ij).

A) B)

Figura 8. Ensayos de germinación sobre A) *L. sativa* y B) *L. perenne*

3.5. TÉCNICAS DE EXTRACCIÓN

3.5.1. Extracción con Soxhlet

La obtención de los extractos de los componentes no voláties se llevan a cabo por una extracción sólido-liquido con disolventes orgánicos en un Soxhlet (Figura 9). Este método consiste en la extracción sucesiva del material sólido colocado en el cuerpo central del aparato con un disolvente orgánico en caliente. El disolvente cae sobre la muestra sólida después de su destilación extrayendo los componentes solubles.

Figura 9. Extractor (Soxhlet) para la obtención de componentes orgánicos no volátiles

3.5.2. Hidrodestilación o destilación por arrastre de vapor

Los aceites esenciales se obtienen mediante destilación por arrastre de vapor de agua, donde el vapor pasa a través de un lecho de material vegetal, extrayendo sus componentes volátiles. Una vez el aceite es arrastrado por el vapor se condensa para su recolección.

A nivel del laboratorio se lleva a cabo por hidrodestilación por el método trampa tipo Clevenger (Figura 10) de acuerdo con el método recomendado por la Farmacopea Europea (http://www.edqm.eu/en/Homepage-628.html).

Figura 10. Hidrodestilador (Clevenger) para la extracción de los componentes volátiles)

3.5.3. Extracción en planta piloto

La extracción semi-industrial del aceite esencial por arrastre de vapor se lleva a cabo en una planta de destilación industrial de acero inoxidable equipada con una cámara de destilación de 100 Kg y un vaso de 500 L (Figura 11). La extracción se realiza en un rango de presión de vapor de 0,5 a 1,0 bar. El agua recolectada después de decantar el aceite esencial se filtra para dar el residuo acuoso (hidrolato) que se extrae con diclorometano (DCM) y se obtiene el correspondiente extracto orgánico.

Figura 11. Planta piloto de arrastre de vapor para a obtención del aceite esencial a partir del material vegetal.

3.5.4. Extracción con fluidos Supercríticos (FSC)

Un fluido supercrítico es cualquier sustancia a una temperatura y presión por encima de su punto crítico termodinámico (Figura 12). Por encima de la temperatura crítica un compuesto puro gaseoso no se puede licuar, independientemente de la presión ejercida. La presión crítica es la presión de vapor del gas a la temperatura crítica. Bajo las condiciones supercríticas la sustancia no es ni un gas ni un líquido pero tiene la propiedad de difundirse a través de los sólidos como un gas, y de disolver los materiales como un líquido. Adicionalmente, puede

cambiar rápidamente la densidad con pequeños cambios en la temperatura o presión. Esto conlleva un proceso de extracción más rápido, eficiente y selectivo que en el caso de la extracción líquido-líquido.

El solvente supercrítico más utilizado es el CO_2, con una baja temperatura supercrítica (31.1 ºC) que evita la degradación térmica de los compuestos extraidos. La baja polaridad de este disolvente supercrítico para extraer compuestos polares se suele solventar con la adición de modificadores, siendo el etanol el más utilizado.

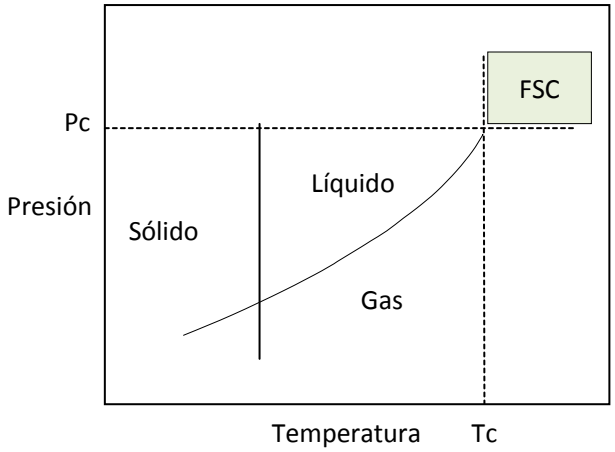


Figura 12. Diagrama de fases

Las extracciones suprecríticas con CO_2 se realizan en una planta piloto (Facultad de Ciencias, Universidad de Zaragoza) (Figura 13) equipada con una bomba de compresión, un filtro, un cilindro de extracción de 1 L, dos colectores de 180 ml, un sistema de refrigeración cilíndrica de 0,5 L, un intercambiador de calor, un sensor de presión y un medidor de flujo (Langa et al., 2009). La presión en los colectores se controla manipulando diferentes válvulas y las bajas temperaturas se consiguen gracias a la acción del sistema de refrigeración.

Las muestras de material se introducen en el cilindro de extracción y son embebidas simétricamente con materiales porosos inertes que permiten un flujo homogéneo de CO_2. Una vez que se alcanza la temperatura de 40º C, la bomba comprime el CO_2 hasta conseguir una presión de 90 bar. Cuando el fluido supercrítico a las condiciones de presion y temperatura deseadas llena el extractor comienza el proceso de extracción.

Figura 13. Planta piloto de extracción de fluidos supercríticos (Universidad de Zaragoza).

El tamaño medio de partícula utilizado es de 0.5 mm, y el flujo de gas a través del extractor de 10.5 l/min (a 25ºC y 1 atm). Los compuestos pesados (ceras) se recojen al final del proceso en el primer colector, mientras que el extracto supercrítico en el segundo colector se pesa a

diferentes intervalos de tiempo para obtener la curva de extracción. Una vez la primera extracción ha finalizado se lleva a cabo una segunda extracción a una presión de 180 bar sobre el mismo material añadiendo 50ml de etanol antes de la extracción.

3.6. TÉCNICAS ESPECTROSCÓPICAS

3.6.1. Actividad optica

La determinación de las actividades ópticas se realiza en un polarímetro Perkin Elmer, modelos 137 y 343 usando la línea D de una lámpara de sodio. Los productos se dissuelven en $CHCl_3$y la medida se realiza en una célula de 1 ml de volumen y 1 dm de longitud (**l**). La concentración se expresa g/100 ml de disolución.

$$[\alpha]_{d=} \alpha \times 100/ l \times c$$

3.6.2. Espectroscopía de infrarrojo (IR)

Los espectros de IR se realizan en un espectrofotómetro Perkin-Elmer modelo 1600/FTIR. Los productos puros se disuelvenen $CHCl_3$ seco y se aplican sobre una célula de cloruro de sodio (NaCl) de 0.5 cm de espesor. Los valores de ν se expresan en cm^{-1}.

3.6.3. Resonancia Magnética Nuclear (RMN)

Los espectros de RMN se realizan en los espectrómetros Bruker Avance 400 MHz y Bruker AMX 500 MHz (Figura 14) (500 y 400 MHz para ^{1}H y 100 y 125 MHz para ^{13}C).

Los productos se disuelven en $CDCl_3$ o MeOD, indicándose en cada caso. Los valores de los desplazamientos químicos (δ) se expresan en partes por millón (ppm), en relación al disolvente empleado como referencia interna, deuterocloroformo (δ_H=7.26 ppm y δ_C=77.16 ppm) o metanol deuterado (δ_H=3.31 ppm y δ_C=49.1ppm). Las constantes de acoplamiento (*J*) se miden en Hertzios (Hz).
Los experimentos bidimensionales de correlación homo y heteronuclear ($^{1}H-^{1}H$, $^{1}H-^{13}C$): COSY, NOESY, HSQC y HMBC se realizan en un espectómetro Bruker AMX 500, utilizando los programas estándar suministrado por la firma Bruker.

3.6.4. Espectrometría de Masas (EM)

Los espectros de masas de baja se realizan en un espectómetro Micromass, modelo Autospec (Figura 14). La técnica empleada fue impacto electrónico con un potencial de ionización de de 70 eV y una temperatura de la fuente de 220 ºC. Los espectros de masas de alta resolución se realizan en un espéctrometro Micromass modelo LCT Premier XE, usando electroespray (ESI) como fuente de ionización de modo positivo y negativo, y en un espectrómetro Micromass Autospec a 70 eV. Para cada producto se indican las fragmentaciones más significativas y su intensidad relativa.

Figura 14. Espectómetro de resonancia magnética (RMN) y masas (EM)

3.7. TÉCNICAS CROMATOGRÁFICAS

3.7.1. Cromatografía en columna (CC)

Para la separación de los compuestos se utilizan columnas cromatográficas, empleando como fase estacionaria gel de sílice Gel de sílice 60 HF_{254} (40-70 µm) de la casa comercial Merck. Como fase móvil se utilizan mezclas de disolventes de n-hexano-AcOEt, CH_2Cl_2-AcOEt, CH_2Cl_2-MeOH, y AcOEt-MeOH en polaridades crecientes.
Las cromatografías flash se realizan en un equipo de cromatografía de media presión (Jones Flash Chromatography) usando columnas preempacadas de 20 g de sílica (ExtraBond Flash OT SI, 40-70µm, 26.8 x 154 mm Scharlau) y una colmna, ajustable, de vidrio con 5 cm de diámetro y 22 centimetro de altura, eluidas con mezclas de disolventes con un régimen isocrático o en gradiente de polaridad.

3.7.2. Cromatografía en Capa Fina (CCF)

Las cromatografías en capa fina (CCF) se realizan empleando cromatofolios de sílica gel de la casa Merck, tipo SIL G-60 F_{254} de 0.20 mm de espesor, con indicador fluorescente a 254 nm.El desarrollo de las cromatografías en columna se siguen por CCF, observando las placas a la luz UV de 254 y 360 nm de longitud de onda. En el sistema de elución se utilizan los mismos disolventes y polaridades que en las columnas cromatográficas.
El revelado de los cromatofolios se realizapor pulverización con *oleum* (disolución de ácido sulfúrico (4%) y ácido acético (80%) en agua destilada) o por inmersión en vainillina (0.5g vanillin, 100ml acido sulfurico/etanol (40:10)), y calentando a una temperatura de 110-130ºC.

3.7.3. Cromatografía líquida a vacío (CLV)

Para este tipo de cromatografía se utiliza como fase estacionaria gel de sílice de grano fino (15-40 µm, Merck). Las dimensiones de las distintas columnas de vidrio utilizadas dependen del peso del extracto y/o fracción de partida. La muestra se coloca en forma de "cabeza de columna", absorbida en gel de sílice más gruesa (0.063-0.200 mm, Merck). La elución se realiza en gradientes de polaridad creciente y como eluyente en la fase móvil se emplean mezclas de n-hexano, AcOEt, CH_2Cl_2 y/o MeOH.

3.7.4. Cromatografía de exclusión molecular (SPH)

Se utiliza como soporteSephadex LH-20 (Pharmacia Fine Chemicals) para la fase estacionaria y una mezcla de disolventes n-hexano:CH_2Cl_2:MeOH en distintas proporciones, dependiendo de la polaridad de la muestra a cromatografiar.

3.7.5. Cromatografía liquida de alta presión (HPLC)

Las cromatografías líquidas de alta presión se realizan en un equipo Beckman Coulter 125P, acoplado a un detector de fotodiodo modelo 168 y un equipo Shimadzu LC-20AD HPLC con detector diodo array.
Las separaciones semipreparativas se llevan a cabo sobre columnas de fase normal, Ultrasphere sílica (Beckman) y 5 SIL (ACE), de 250 mm x 10 mm y 5 µ de tamaño de partícula. Dependiendo de la cantidad de muestra, se utilizan también columnas preparativas de silica Interstil de 20 mm x 250 mm y 10 µ de tamaño de partícula. Las muestras se filtran previamente con filtros de 0.45 µm de tamaño de poro y se emplean disolventes de grado HPLC.

3.7.6. Cromatografía de gases acoplada a espectrometría de masas (GC-MS)

La determinación cualitativa y cuantitativa de los compuestos volátiles presentes en los extractos, aceites esenciales y fracciones volátiles se analiza por cromatografía de gases-espectrometría de masas (GC-MS), en un cromatógrafo de gases Agilent modelo 5973N acoplado a un detector de masas con fuente de ionización por impacto electrónico (IE) a 70eV (Figura 14).

Con este equipo se ha utilizado una columna capilar HP-1 (fase ligada de metil silicona) de Hewlett-Packard: 25 m de largo, 200 µm de diámetro interno y 0.2 µm de espesor de fase. Las condiciones utilizadas han sido: split (30:1), temperatura del inyector 260ºC, temperatura de la columna 70ºC, calentando hasta 270ºC a 4ºC/min. Los espectros de masas y el tiempo de retención han sido utilizados para identificar los compuestos por comparación con los encontrados en la base de datos Wiley (Wiley 275 Mass Spectra Database, 2001) mientras que para la cuantificación se han utilizado los % del área de los picos obtenidos en los cromatogramas.

3.7.7. Cromatografía líquida de alta resolución acoplada a espectrometría de masas (HPLC-MS).

Los extractos no volátiles se analizan por HPLC-MS en un equipo Shimadzu con bomba LC-20AD acoplado a un espectrómetro de masas con cuadrupolo como analizador (LCMS-2020 QP) y usando una interfase e ionización por electroespray (ESI). (Figura 15)
Para la separación se utiliza una columna Teknokroma, Mediterranea Sea_{18} column (250 mm x 4.6 mm, 5 µm de tamaño de partícula) con una pre-columna analítica ACE3 C18.
Las soluciones stock de los extractos se analizan a 0.25 µg/µl y los compuestos puros a 0.05 µg/µl (patrones) disueltos en 100% MeOH con una inyección de muestra de 10 µl. Todos los disolventes utilizados son de grado HPLC-MS.

Figura 15. Cromatógrafo de gases y líquido acoplados a espectrometria de masas (GC-MS y HPLC-MS).

4. TRABAJOS PUBLICADOS / EN ELABORACIÓN

4.1. Chemical composition and biological effects of essential oils from *Artemisia absinthium* L. cultivated under different environmental conditions

Maria Bailen, Luis F. Julio, Carmen E. Diaz, Jesus Sanz, Rafael A. Martínez-Díaz, Raimundo Cabrera, Jesus Burillo, Azucena Gonzalez-Coloma

Industrial Crops and Products (**2013**), *49*, 102-107.
doi:10.1016/j.indcrop.2013.04.055

Abstract

The objective of this study was the valorization of the essential oils from Spanish *Artemisia absinthium* domesticated plants from Teruel and Sierra Nevada (Spain). These populations were experimentally cultivated in the field and under controlled conditions. The insect antifeedant properties of their essential oils collected yearly from two locations were tested against *Spodoptera littoralis*, *Myzus persicae* and*Rhopalosiphum padi*. Additionally we studied their phytotoxic, antifungal and antiparasitic effects. The oils from cultivated *A. absinthium* were characterized by the presence of *cis*-epoxyocimene, chrysanthenol, and chrysanthenyl acetate. The variations observed in oil composition were mostly quantitative but also qualitative. (Z)-2,6-Dimethyl-5,7-octadien-2,3-diol has been isolated and identified by NMR. Among the oil samples, these rich in *cis*-epoxyocimene and sesquiterpenes were the most active ones against *S. littoralis*. (Z)-2,6-Dimethyl-5,7-octadien-2,3-diol showed moderate activity against *S. littoralis*. The strongest antifeedant effects were found for commercial *A. absinthium* oil samples rich in thujones and sabinyl acetate. *F. oxysporum* and *F. solani* were affected by oils from cultivated *A. absinthium* and commercial oil samples. Oils from cultivated *A. absinthium* showed antiparasitic effects against *Leishmania infantum* and *Trypanosoma cruzi* with better results than the commercial samples.

Keywords: *Artemisia absinthium*, cultivated, essential oil, antifeedant, antifulgal, antiparasitic.

1. Introduction

The genus *Artemisia* is a genus that belongs to the family Compositae (Asteraceae) and consists of about 500 species distributed through the world (Bora and Sharma, 2011).*Artemisia absinthium* L. is an aromatic and medicinal plant of ethnopharmacological interest (Bora and Sharma, 2010 and Lachenmeier, 2010). The composition and biological effects of the essential oil (EO) of *A. absinthium* has been widely studied. *A. absinthium* EOs had antimicrobial (Baykan Erel et al., 2012, Gandomi Nasrabadi et al., 2012 and Juteau et al., 2003) and antiprotozoal effects against *Leishmania aethiopica*and *L. donovani* (Tariku et al., 2011). In addition, thujone-rich oils have been shown to have acaricidal (Chiasson et al., 2001), insecticidal (Kaul et al., 1978, Kordali et al., 2006 and Umpiérrez et al., 2012) and fungicidal effects (Umpiérrez et al., 2012) and myrtenol-rich oils repelled fleas, flies, mosquitoes (Erichsen-Brown, 1979) and ticks (Jaenson et al., 2005).

Among the major EO components reported are α and β-thujone (Carnat et al., 1992, Chialva et al., 1983, Geszprych et al., 2010 and Umpiérrez et al., 2012), myrcene, trans-sabinyl acetate (Geszprych et al., 2010, Judzentiene et al., 2012, Karp and Croteau, 1982, Lopes-Lutz et al., 2008 and Sharopov et al., 2012), β-pinene, (Kordali et al., 2005), 1,8-cineole (Kordali et al., 2005 and Tehrani et al., 2012), camphor (Tariku et al., 2011), *cis*-epoxyocimene (Chialva et al., 1976), chrysanthenil acetate (Chialva et al., 1983,Geszprych et al., 2010 and Kordali et al., 2005), sabinene (Baykan Erel et al., 2012,Geszprych et al., 2010 and Kordali et al., 2005), myrtenol (Erichsen-Brown, 1979 and Jaenson et al., 2005), bornyl acetate (Pino et al., 1997), artemisia ketone, linalool, hydrocarbon monoterpenes (Kordali et al., 2005), sesquiterpene lactones (Leung, 1980 and Martín et al., 2011a) and mixtures of some of these components (Carnat et al., 1992, Chialva et al., 1983 and Geszprych et al., 2010), depending on the plant origin.

A. absinthium is abundant in the mountains of Spain as ruderal species and it is used as a medicinal remedy. There are seven chemotypes described in the Iberian Peninsula and some of them are thujone-free (Ariño et al., 1999). Therefore, the use of *A. absinthium*based on the collection of wild populations can result in variable compositions of the extracts. As part of an ongoing project on the valorization of native plants and the sustainable production of botanical biopesticides, Spanish populations of wormwood have been domesticated for experimental cultivation in the field and under controlled conditions (Burillo, 2009, Gonzalez-Coloma et al., 2012, Martín et al., 2011a and Martín et al., 2012) to generate agronomic (Burillo, 2009) and chemical data. The biological effects (insect antifeedant action and antioxidant effect) and constituents of the ethanolic extracts (OSE) of these two populations have been described as a function of cultivation method, location and time, with the sesquiterpene lactone hydroxypelenolide being the major component followed by the flavones artemetin, and casticin. Casticin concentrations correlated with the antifeedant and antioxidant effects of these wormwood extracts. Furthermore, optimized supercritical fluid extracts of cultivated Spanish *A. absinthium* showed an improvement in the yield of several mono- and sesquiterpenes and were more active than the traditional extracts (EO, OSE) against insects (Martín et al., 2011a and Martín et al., 2012).

Here we report on the chemical composition of the essential oils of domesticated *A. absinthium* plant samples collected yearly from two locations and from clonic plants cultivated under controlled conditions. One unidentified major component was isolated and identified by NMR experiments as the monoterpene (Z)-2,6-dimethylocta-5,7-dien-2,3-diol. The insect antifeedant properties (*Spodoptera littoralis, Myzus persicae and Rhopalosiphum padi*), antifungal (*Fusarium* spp.) and phytotoxic (*Lactuca sativa, Lolium perenne*) effects of the EOs were tested. Additionally we studied their antiparasitic effects on *Trypanosoma cruzi* and *Leishmania infantum*.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were determined in $CHCl_3$ at room temperature using a Perkin-Elmer 137 polarimeter. IR spectra were taken on a Perkin-Elmer 1600 FT spectrometer. UV spectra were measured on a Hewlett-Packard HP-8254-A. NMR spectra were measured on a Bruker AMX2 500 MHz spectrometer with pulsed field gradient using the solvent as internal standard ($CDCl_3$, at δ_H 7.26 and δ_C 77.0). The programs used in two-dimensional (2D) NMR experiments (HMBC, HSQC, COSY, and NOESY) were those furnished with the manufacturer's software. EIMS and exact mass measurements were recorded on a Micromass Autospec instrument at 70 eV. Preparative and semipreparative HPLC was carried out with a Beckman Coulter 125P equipped with a diode-array detector 168 and preparative Interstil silica 20 mm × 250 mm, 10 µm particle size and semipreparative Ultrasphere silica 10 mm × 250 mm, 5 µm particle size columns. Silica gel 60 F_{254} (Merck, art. 105715) and Sephadex LH-20 (Pharmacia) were used for column chromatography. Compounds were visualized on TLC with Oleum reagent.

2.2. Plant material and cultivation

Plant material for field cultivation was selected from a wild population growing in Teruel (Spain). The individuals for field cultivation were obtained from seeds. The experimental fields were located in Barrio de San Blas, Teruel, Spain (T) and Ejea de los Caballeros, Zaragoza, Spain (E). A detailed description of these fields and the cultivation parameters has been published (Burillo, 2009). Flowering plant samples from 30 randomly selected plants were collected yearly and processed for EO extraction. The EOs analyzed corresponded to crops collected for 5 consecutive years (E1-E5 and T2-T5, Table 1).

The individuals for growth chamber, aeroponic orgreenhouse cultivation (SN) were obtained from a population from the nursery at the Sierra Nevada National Park (Granada) and cultivated as described for one year (Gonzalez-Coloma et al., 2012). Aerial parts of the growth chamber, green house and aerponically grown plants were collected for extraction (SNC, SNI and SNA samples respectively). For comparison purposes, a wild population V (Villacampa, Huesca, Aragón, 2006) and commercial *A. absinhium* EO samples H of different geographical origin (H1, commercial essential oil; H1.2, waste oil fraction rich in thujones and terpenes and H1.3, commercial oil fraction for the agrifood industry, rich in sesquiterpenes, esters and azulenes; Hausmann Aromatic S.A., San Andrés de la Barca, Barcelona, Spain) were also included in the study. A summary of all the samples studied is shown in Table 1.

2.3. Essential oil extraction and analysis

Plant samples (100 g) were distilled in a Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia (7th Ed., 2010). The essential oils were analyzed by GC-MS using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, California, USA) coupled to an Agilent 5973N mass detector (electron ionization, 70 eV) (Agilent Technologies, Palo Alto, California, USA) and equipped with a 25 m × 0.20 mm i.d. capillary column (0.2 µm film thickness) HP-1 (methyl silicone bonded) (Hewlett-Packard). Working conditions were as follows: split ratio (30:1), injector temperature, 260 °C; temperature of the transfer line connected to the mass spectrometer, 280 °C; column temperature 70 °C for 5 min, then heated to 270 °C at 4 °C min^{-1}. EI mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001). Quantitative data were obtained from the TIC peak areas without the use of response factors.

2.4. Compound isolation

A bulk acetone extract (260 g, 25.8% yield) of the E2 sample (1000 g plant dry weight, 48.9% water content) was chromatographed on a vacuum liquid column (VLC) and eluted with n-hexane:EtOAc:MeOH gradients to give 8 fractions. Fraction 3 (3.9 g, 0.39%), was further chromatographed on Sephadex LH20, Si-gel column and preparative HPLC to give the monoterpene (Z)-2,6-dimethylocta-5,7-dien-2,3-diol (25.2 mg, 2.5 × 10^{-3}%).

^{1}H-NMR, MS and ^{13}C-NMR spectra of (Z)-2,6-dimethylocta-5,7-dien-2,3-diol coincided with those reported by Tsankova and Bohlmann (1983) (Fig. 1).

2.5. Insect bioassays

S. littoralis M. persicae and *R. padi* colonies were reared on artificial diet, bell pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) plants, respectively, and maintained at 22 ± 1 °C, >70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber. The bioassays were conducted with newly emerged *S. littoralis* L6 larvae or ten*M. persicae* /*R. padi* adults as described by Burgueño-Tapia et al. (2008). Thujone (Sigma) was included as a positive control.

2.6. Antifungal activity

Fusarium moniliforme (Sheldon) [CECT2152], *F. oxysporum* fs. *lycopersici* (Scheldt) [CECT2715] and *F. solani* (Mart) [CECT2199] were provided by the *Colección Española de Cultivos Tipo* (CET). Antifungal activity was analyzed as mycelial growth inhibition by a modified agar-dilution method which includes the addition of 0.05 mg/mL of methyltetrazolium salts (MTT). Colonies grown on Petri dishes incubated for 48 h were digitalized and measured (SIGMASCAN). Percent inhibition (%*I*) was calculated as: $\%I = (C - T/C) \times 100$, where *C* is the diameter of the control colonies and *T* is the diameter of the test colonies. The active

compounds were tested in a dose-response experiment to calculate their relative potency (EC_{50} values, the effective dose to give 50% inhibition).

2.7. Antiparasitic activity

Leishmanicidal activity was assayed on promastigote forms of *L. infantum* PB75 strain, cultured at 28 °C in RPMI medium supplemented with 10% fetal calf serum. Parasites in logarithmic growth phase were distributed in 96-well flat bottom plates. The essential oils were tested at several concentrations (800, 400, and 100 μg/ml) for 72 h. Amphotericin B was used as reference drug and parasite viability was analysed by modified MTT colorimetric assay (Gonzalez-Coloma et al., 2012). The activity was calculated as % growth inhibition as folows: $\%GI = 100 - [(A_p - A_b)/A_c - A_b) \times 100]$, A_p being the absorbance of problem wells (treated), Ac the absorbance of control wells (not treated) and A_b the absorbance of blank wells (culture medium and vehicle only).

Trypanocidal activity was assayed on epimastigote forms of *T. cruzi* Y strain, cultured in LIT medium. Parasites in logarithmic growth phase (from an initial culture with 2×10^6epimastigotes/ml) were distributed in 96-well flat bottom plates. Each well was treated with increasing concentrations of the essential oils for 96 hours as described above. Nifurtimox was used as the reference drug and parasite viability was analyzed by a modified MTT colorimetric assay method (Gonzalez-Coloma et al., 2012). The activity was calculated as described above for *Leishmania*.

2.8. Statistical analysis

The antifeedant effects were analyzed for significance by the non parametric Wilcoxon signed-rank test. The dependence of antifeedant effects with the composition of essential oils from samples E, T and V (Tabel 1) was studied by multiple linear regression (Statgraphic Plus 5.1). The stepwise regression mode was chosen in order to select the independent variables (component concentrations) with a significant correlation with the biological activity.

The antifungal relative potencies (EC_{50} values, the effective dose to give 50% inhibition) were determined from linear regression analysis (% inhibition on log dose, SPSS.11).

3. Results and discussion

3.1. EO composition

A total of fifteen wormwood essential oils were analyzed (Table 1). Table 2 and Table 3 show their chemical composition.

The EOs from cultivated *A. absinthium* were characterized by the presence of *cis*-epoxyocimene (**4**), chrysanthenol (**7**), and chrysanthenyl acetate (**11**) among others. These compounds were also found in supercritical extracts of *A. absinthium* E samples in variable concentrations (Martín et al., 2011b) and have been described in EOs of this plant (Carnat et

al., 1992, Chialva et al., 1976, Geszprych et al., 2010, Judzentiene and Budiene, 2010 and Kordali et al., 2005).

cis-Epoxyocimene (**4**) was the major component of E1-E3 (22.8–51.5%) and T2-T4 (30.5–44.8%) EOs (Table 2) and its abundance were lower for E4, E5 and T5. Among the SN samples, **4** was the major component of SNA (75.1%), was present in SNC (7.8%), and absent in SNI. The major component of the wild sample V was also **4**(45.7%). Compound **7** was present in all of the EOs studied in variable amounts (4.4–21.4%). Chrysanthenyl acetate (**11**) was present in all the field cultivated samples (E and T) (1.5–32.2%). SNI and SNC had low amounts of **11** (1.5 and 2.5% respectively) in contrast to the wild sample V (32.2% of **11**). Linalool oxide (**14**) was found in E1-E3 (34.3–0.5%), T2-T5 (2.9–24.1%) and was the major component of SNI (54.1%). Among the sesquiterpenes (**14**–**17**), caryophyllene (**16**) was found in E1-3, T2-5 (1.2–3.4%). Compounds **18** and **19**, increased with time for the E samples (0.4 to 5.4 and 1.0 to 4.6% respectively). Diterpenes **21** and **22** increased in E4 (7.5 and 10.6% respectively). For the SN samples, **16** (1.3–5.7%) and **19** (0.9–5.6%) were present in all of them. Diterpenes were only found in SNC (**21** and **22**) and SNI (**20** and **21**) in low amounts.

Table 1. *A. absinthium* samples of different origins (Teruel, T; Sierra Nevada, SN, Villacampa, V and commercial, H), cultivated under different conditions (field and controlled environment) in different locations (Ejea and Teruel) for four years.

Origin	Crop	Cultivatiated					Wild	Commercial
		Growth chamber	Greenhouse	Aeroponic	Field			
					Ejea[a]	Teruel[b]		
SN[c]	1	SNC	SNI	SNA				
T[b]	1				E1	na		
	2				E2	T2		
	3				E3	T3		
	4				E4	T4		
	5				E5	T5		
V[d]	-						V	
H[e]	-							H1 H1.2 H1.3

[a]Ejea de los Caballeros (Zaragoza): 42º07’16’’N, 1º08’20’’O, 398m
[b]San Blas (Teruel): 40º21’30’’N, 1º10’45’’O, 923m
[c]Sierra Nevada (Granada): 37º05’33’’N, 3º23’04’’O, 2083m
[d]Villacampa (Huesca): 42º30’32’’N, 0º21’21’’O, 759m
[e]Hausmann SA; na, not available

Some compounds were only found in one or two samples (Table 2). Camphor (**6**) was present in E2 and T4 (6.7 and 1.9%), linalyl propionate (**7**) in E3 and T5 (1.04 and 6.2%), linalyl acetate (**9**) in E3 (4.2%), bornyl acetate (**10**) in SNC (15.1%) and thymol (**13**) in T1 (10.0%). Bornyl acetate (**10**) has been found as a major constituent of *A. absinthium* oil from Cuba (Pino et al., 1997).

(Z)-2,6-dimethylocta-5,7-dien-2,3-diol (**12**) was found in E4 and E5 (the last two crops from E) in relatively large amounts (30 and 54.6%) (Table 2). Compound **12** might be the reaction product of the hydrolysis of *cis*-epoxiocimene (**4**) (an extraction artifact) or biosynthetized by the plant from compound **4**. Compound **12** has been isolated from an acetone extract of E2 and identified by NMR (Fig. 1). (Z)-2,6-dimethylocta-5,7-dien-2,3-diol (**12**) has been reported in *Passiflora quadrangularis* (Osorio et al., 2002), in the glandular haired accessions of *Medicago* spp. resistant to the alfalfa weevil (Core et al., 1996) and in the aerial parts of *Aster bakeranus* (Tsankova et al., 1983).

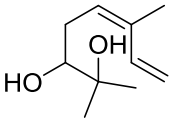


Fig. 1. (Z)-2,6-dimethylocta-5,7-dien-2,3-diol (**12**).

Table 2. Chemical composition of the different *A. absinthium* samples (abundance: % area).

No.	Compound	Field, Ejea					Field, Teruel				Controlled			Wild
		E1	E2	E3	E4	E5	T2	T3	T4	T5	SNC	SNI	SNA	V
1	α-Pinene	0.4	2.0	0.5										
2	1,8-Cineole	1.02		2.5					1.2	0.8				0.6
3	Linalool	2.6	4.7	6.1		1.6	2.0	4	2.4	4.4			0.4	
4	*cis*-Epoxyocimene	22.8	59.9	41.8	1.1	2.0	30.5	31.0	44.8	4.3	7.8		75.1	45.7
5	*Trans*-epoxyocimene	2.51		4.0			2.3	2.1	2.7					3.3
6	Camphor		6.9							1.9				
7	Chrysanthenol	10.1	13.2	12.9	9.3	19.3	5.9	8.1	4.4	17.0	12.3	21.4	10.6	11.1
8	Linalyl propionate			1.04						6.2				
9	Linalyl acetate			4.2										
10	Bornyl acetate										15.1			
11	Chrysanthenyl acetate	9.8	2.2	11.6	2.4	4.4	10.5	17.2	16.8	19.4		1.5	2.5	32.2
12	$C_{10}H_{18}O_2$(Z)-2,6-dimethylocta-5,7-dien-2,3-diol				30.0	54.6								
13	Thymol (Phenol, 5-methyl-2-(1-methylethyl)-						10.0							
14	*cis*-Linalool oxide (furanoid)	34.3	6.3	0.5			2.9	24.1	12.2	16.7		54.1		
15	trans-Linalool oxide (furanoid)	1.3						1.2						
16	Caryophyllene	1.2	2.0	1.3			3.4	1.3	2.0	2.9	5.7	1.3	1.8	
17	β-Selinene	0.5	1.7	1.9			1.0	0.7	0.5	1.2			0.7	
18	Caryophyllene oxide	0.4	1.2	1.1	2.6	5.4		0.5	1.3	3.7		4.3	0.7	
19	$C_{10}H_{18}O$ sesquiterpene	1.0	0.5	1.5	2.7	4.6	3.8	0.9	2.1	6.6	4.6	5.6	0.9	
20	Diterpene $C_{20}H_{28}O$	tr		tr	7.5		tr	tr	tr	tr		1.2		
21	Diterpene $C_{20}H_{30}O$	1.1	1.7	0.9			0.6	0.8	tr	tr	4.1	0.9		
22	Diterpene $C_{20}H_{28}O$	0.1		0.3	10.6		0.4	tr	tr	1	1.3			

The variations observed in *A. absinthium* EO composition were mostly quantitative but also qualitative, with the samples grown in controlled environments (SN) showing a more simplified composition than the field ones (E and T) except for E4 and E5. Moreover, the wild sample V showed fewer components than the cultivated ones. Chemical variations have been described for *A. absinthium* essential oils from plants cultivated *in vitro* (absence of monoterpenes and thujones and higher amounts of heavier compounds), in the greenhouse and in the field (presence of high amounts of monoterpenes and thujones) (Gholami et al., 2005).

Two chemotypes have been described from the Iberian Peninsula (Ariño et al., 1999); *cis*-epoxyocimene type (with more than 50% of this compound) which was predominant in all the populations and a *cis*-epoxyocimene + chrysanthenyl acetate type (with 25-65% of *cis*-epoxyocimene and 15–50% of chrysanthenyl acetate). E, T, and V oils fitted the *cis*-epoxyocimene + chrysanthenyl acetate type; SNA the *cis*-epoxyocimene type, while the other SN oils did not follow that classification. Furthermore, if linalool oxide derived from *cis*-epoxyocimene as suggested (Blagojevic et al., 2006), SNI could be included in the first group. None of the plants analyzed contained thujone as previously shown by Ariño et al. (1999). Wormwood essential oils from other locations had *cis*-chrysanthenol as the main component (Carnat et al., 1992 and Judzentiene and Budiene, 2010), *cis*-epoxyociene and β-thujone/cis-chrysanthenyl acetate among other compositions (see Blagojevic et al., 2006).

Table 3. Chemical composition of commercial oil (H1) and its fractions (H1.2, H1.3) (abundance: % area).

No.	Compound	H1	H1.2	H1.3
1	Sabinene	1.8	2.5	
2	Limonene	3.1	4.6	
3	Linalool	3		
4	α-Thujone	10.4	22.3	2.1
5	β-Thujone	40.2	57.1	6.6
6	*cis*-epoxyocimene	1.8	2	
7	Thujol	0.3	0.3	0.5
8	Phellandrene epoxide		1.3	
9	Sabinol	1.6		2.1
10	Lavandulol	0.9	0.5	1.1
11	α-Terpineol	0.5	0.3	0.3
12	Sabinyl acetate	27.2	4.2	59.7
13	Caryophyllene	1		3.2
14	β-Selinene	0.8		2.7

Table 3 shows the chemical composition of the commercial oil samples (H1) and its fractions (H1.2, H1.3). The chemistry of the H1 oil agrees with the G-type (Ariño et al., 1999), with thujones and metabolically related compounds such as sabinyl acetate as the major components. H1.2 fraction, rich in low molecular weight compounds, had the largest amount of thujones while H1.3, rich in heavier compounds (sesquiterpenes and esters), had higher content in sabinyl acetate. The oil H1 showed a chemical profile containing a mixture of both fractions (H1.2 and H1.3).

3.2. Antifeedant effects

Table 4 shows the antifeedant effects of the different *A. absinthium* oils studied here. Among the cultivated samples, E2, SNA and T4 rich in *cis*-epoxyocimene (**4**) (>45%) were the most active ones against *S. littoralis*. Sample V with 45.7% of **4** but lacking sesquiterpenes was not active against this insect, suggesting synergistic effects for E2, SNA and T4. Isolated (Z)-2,6-dimethyl-5,7-octadien-2,3-diol (**12**) showed moderate activity against *S. littoralis* (50 μg/cm^2), while E4 and E5 had lower effect (30–55% of the diol **12**). Moderate-low effects (%SI > 60) were found for E2, T3 and E3 against the aphids. The strongest antifeedant effects were found for the commercial samples H, with sample H1.3 rich in sabinyl acetate being active against the three insect species followed by H1.2 (thujones and monoterpenes). Thujone was more active than the H samples. Thujone and H1.2 orally dosed to *S. littoralis* did not affect larval growth or ingestion (data not shown), suggesting metabolic detoxification. Thujone is a neurotoxic insecticide (Ratra et al., 2001). Therefore, the antifeedant effects of the H samples could be related to the presence of this compound.

Table 4. Antifeedant effects of *A. absinthium* essential oils from cultivated (E, T and SN) wild (V) and commercial (H) samples (100 μg/cm^2).

Sample	R. padi(%SI [a])	M. persicae(%SI [a])	S. littoralis(%FI [b])
E1	26 ± 9	40 ± 8	34 ± 15
E2	67* ± 5	55* ± 9	81* ± 10
E3	26 ± 7	61* ± 8	50 ± 21
E4	31 ± 7	16 ± 6	21 ± 9
E5	40 ± 7	34 ± 6	21 ± 13
12	nt	25 ± 6	62* ± 17
T2	25 ± 6	22 ± 7	39 ± 17
T3	66* ± 7	49 ± 7	49 ± 20
T4	30 ± 9	42 ± 8	58 ± 19
T5	34 ± 6	26 ± 8	48 ± 22
SNC	39 ± 8	43 ± 8	48 ± 10
SNI	25 ± 7	26 ± 8	54 ± 20
SNA	55 ± 6	32 ± 9	70 ± 6
V	39 ± 6	42 ± 6	26 ± 17
H1	17 ± 7	29 ± 9	89* ± 3
H1.2	57* ± 7	64* ± 9	84* ± 5
H1.3	71* ± 7	77* ± 5	77* ± 5
Thujone	72 ± 20	85* ± 16	100* ± 0

a Percent settling inhibition
b Percent feeding inhibition
* $p < 0.05$, Wilcoxon Paired Rank Test.

The result of a step-wise multiple regression performed between the oil composition of samples E and T and V and their antifeedant effects indicated that such activity against*M. persicae* can be partially explained by linalool (35.8%, R^2 adjusted, $p = 0.031$), and against *S. littoralis* by *cis*-epoxyocimene (36.8%, R^2 adjusted, $p = 0.028$). There was not significant relationship between any of the components and the antifeedant effects against *R. padi*, suggesting synergistic effects. Linalool has been reported as an antifeedant and repellent to *M.*

persicae (Rodilla et al., 2008) while the (+)-(R)-enantiomer of (E)-6,7-epoxyocimene has been previously identified as a pheromone emitted by the male fruit-spotting bug *Amblypelta nitida* (see Agnaniet et al., 2011).

Moreover, phytotoxic effects of the EOs were tested against *Lactuca sativa* and *Lolium perenne* seeds without significant results (data not shown).

Supercritical extracts of the cultivated *A.absinthium* (E and SN) showed strong antifeedant and insecticidal effects against *S. littoralis* and *Rhopalosiphum padi* (Martín et al., 2011a and Martín et al., 2012), indicating that the extraction method can play an important role when evaluating the potential applications of *A. absinthium* extracts. Other wormwood EOs and their major components (camphor, 1,8-cineole, terpinen-4-ol, borneol, bornyl acetate and α-terpineol) have shown insecticidal effects against the granary weevil *Sitophilus granarius* (Coleoptera) (Kordali et al., 2006). Thujone rich *A. absinthium* oils from Uruguay had insecticidal effects against *Tuta absoluta* and*Trialeuroides vaporariorum* (Umpiérrez et al., 2012).

3.3. Antifungal activity

The results of the antifungal assays of wormwood essential oils against three species of*Fusarium* are shown in Table 5. *F. oxysporum* and *F. solani* were affected by E1, T2 and H1.3, while H1.2 was active on the three species. ($ED_{50} \cong 0.5$ μg/ml). E1 and T2 have in common significant amounts of *cis*-epoxyocimene (**4**), H1.2 had the highest concentration in β-thujone and H1.3 in sabinyl acetate.

Table 5. Antifungal effects of *A. absinthium* essential oils from cultivated (E, T and SN) wild (V) and commercial (H) samplesagainst *Fusarium* sp. ED_{50} (μg/ml) (upper and lower 95% confidence limits).

Sample	*F. moniliforme*	*F. oxysporum*	*F. solani*
E1	>1	0.5 (0.4, 0.)	0.6 (0.2, 0.9)
E2	>1	>1	>1
E3	>1	>1	>1
E4	>1	0.8 (-0.1, 1.2)	>1
E5	>1	>1	>1
T2	0.8 (0.7, 0.9)	0.58 (0.5, 0.7)	0.6(0.5, 0.7)
T3	>1	>1	0.8 (0.5, 1.0)
T4	>1	0.9 (0.6, 1.2)	>1
T5	>1	0.9 (0.7, 1.2)	0.9 (0.7, 1.2)
SNC	>1	>1	>1
SNI	>1	0.8 (0.5, 1.0)	>1
V	>1	>1	>1
H1	>1	>1	>1
H1.2	0.5 (0.5, 0.6)	0.5 (0.5, 0.5)	0.4 (0.4, 0.5)
H1.3	>1	0.6 (0.4, 0.7)	0.5 (0.4, 0.7)

A. absinthium EO from France containing (*Z*)-epoxyocimene and chrysanthenyl acetate as major components inhibited the growth of both the yeasts *Candida albicans* and*Saccharomyces cerevisiae* var. chevalieri (Juteau, 2003). Wormwood essential oil from a Turkish population, whose main components are camphor, 1,8-cineole and chamazulene, has been described as fungicidal against 34 species of fungi including *F. solani* and *F. oxysporum* (Kordali et al., 2005). Furthermore, *A. absinthium* EO from Uruguay rich in thujone showed antifungal effects against *Alternaria* sp. and *Botrytis cinerea* (Umpiérrez et al., 2012). However, further studies are needed to identify the compound/s responsible for the antifungal effects of *A. absinthium* EOs.

3.4. Antiparasitic activity

Table 6 shows the antiparasitic and cytotoxic effects of wormwood essential oils against*L. infantum* and *T. cruzi* respectively.

For this assays we selected the essential oils with insecticidal activity (T2, E2, SNC and H1.2). E2 and SNC EOs showed antiparasitic effects against *Leishmania infantum* at all the lowest dose tested, while T2 and H1.2 were at 800 and 400 µg/ml. These EOs, except H1.2 (inactive), were active against *Trypanosoma cruzi* at 800 and 400 µg/ml, with SNC being active al 100 µg/ml.

Table 6. Leishmanicidal and trypanocidal effects (% mortality) from A. absinthium essential oil from different populations.

Essential oil (µg/ml)	L. infantum			T. cruzi		
	800	400	100	800	400	100
E2	83 ± 0.2	88 ± 0.6	91 ± 0.6	94 ± 1.1	92 ± 1.5	15 ± 3.0
T2	79 ± 0.1	91 ± 0.6	0	85 ± 1.8	89 ± 0.5	35 ± 8.4
SNC	90 ± 0.8	96 ± 0.3	80 ± 0.9	95 ± 0.6	99 ± 0.2	71 ± 2.8
H1.2	89 ± 2.6	84 ± 1.9	28[a] ± 9.5	12 ± 4.5	0	0[a]

[a]Dose of 200 µg/ml.
[b]Dose of 800 µg/ml.

The main components of the EOs tested (*cis*-epoxyocimene, chrysanthenol, bornyl acetate and thujone) have not been reported as antiparasitary agents against*Leishmania* and *Trypanosoma*. Therefore, further studies are needed to identify the compound/s responsible for the antiparasitic effects of *A. absinthium* EO.

4. Conclusions

The EOs from experimentally cultivated *A. absinthium* were characterized by the presence of *cis*-epoxyocimene, chrysanthenol, and chrysanthenyl acetate among others. The variations observed in EO composition were mostly quantitative but also qualitative. (Z)-2,6-dimethylocta-5,7-dien-2,3-diol has been isolated and identified by NMR. Among the cultivated samples, these rich in *cis*-epoxyocimene and sesquiterpenes were the most active ones against *S. littoralis*. Isolated (Z)-2,6-dimethyl-5,7-octadien-2,3-diol showed moderate activity against *S. littoralis*. The strongest antifeedant effects were found for the commercial samples, rich in thujones and sabinyl acetate. *F. oxysporum* and *F. solani* were affected by EOs from cultivated *A. absinthium*and commercial oil samples. EOs from cultivated *A. absinthium* showed antiparasitic effects against *Leishmania infantum* and *Trypanosoma cruzi* with better results than one of the fractions isolated from the commercial oil (H1.2). Therefore, thujone free cultivated *A. absinthium* plants have the potential to be developed for the production of biopesticides and antiparasitic agents.

Acknowledgements

This work was supported by grant CTQ2009-14629-C02-01 (Spain). M. Bailén and L.F. Julio thank CSIC (Spain) for a predoctoral JAE fellowship.

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4.2. Chemical and biocidal characterization of two cultivated *Artemisia absinthium* populations with different domestication levels

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Industrial Crops and Products (**2015**), *76*, 787-792.
doi:10.1016/j.indcrop.2015.07.041

Abstract

The objective of this study was the characterization and valorization of the essential oil from a domesticated *Artemisia absinthium* population (Teruel, Spain), and its comparison with another one (Sierra Nevada, Spain) undergoing the domestication process. These populations are being experimentally cultivated in the same field since 2008. We studied their biomass and essential oil production (Clevenger hydrodistillation, HD and semi-industrial vapor-pressure, VP). The domesticated population showed lower chemical variation and higher biomass and essential oil yields, allowing for the registration of a new plant variety. The observed variations in oil composition (HD, VP) between the two populations were mostly quantitative. The oils were characterized by the presence of *cis*-epoxyocimene, (-)-*cis*-chrysanthenol, chrysanthenyl acetate, linalool and *trans*-caryophyllene. The insect antifeedant (*Leptinotarsa decemlineata*, *Spodoptera littoralis*, *Myzus persicae* and *Rhopalosiphum padi*) and antifungal (*Fusarium* spp. and *Botrytis cinerea*) effects of their oils were also tested. All VP extracts showed strong antifungal effects and the active antifungal compounds have been identified.

Keywords

Artemisia absinthium, Domestication, Hydrodistillation, Vapor pressure, Antifungal, cis-chrysanthenol.

1. Introduction

Artemisia absinthium L. is a perennial aromatic and medicinal plant of ethno pharmacological interest (Bora and Sharma, 2010 and Lachenmeier, 2010) the composition and biological effects of the essential oil and the extracts of *A. absinthium*has been widely studied. Among the major *A. absinthium* essential oil components reported are α and β-thujone, myrcene, trans-sabinyl acetate, β-pinene, 1,8-cineole, camphor, *cis*-epoxyocimene, chrysanthenyl acetate, sabinene, myrtenol, bornyl acetate, artemisiaketone, linalool, hydrocarbon monoterpenes and sesquiterpene lactones (Pino et al., 1997, Jaenson et al., 2005, Kordali et al., 2005, Geszprych et al., 2011, Martín et al., 2011, Baykan Erel et al., 2012, Judzentiene et al., 2012, Sharopov et al., 2012, Tehrani et al., 2012 and Umpiérrez et al., 2012), in variable concentrations depending on the plant origin (Chialva et al., 1983, Carnat et al., 1992, Geszprych et al., 2011 and Bailen et al., 2013). In addition, thujone-rich oils have been shown to have acaricidal (Chiasson et al., 2001), insecticidal (Kaul et al., 1978, Kordali et al., 2006 and Umpiérrez et al., 2012) and fungicidal effects (Umpiérrez et al., 2012) and myrtenol-rich oils repelled fleas, flies, mosquitoes (Erichsen-Brown, 1979) and ticks (Jaenson et al., 2005).

As part of an ongoing project on the valorization of native plants for the sustainable production of botanical biopesticides, a Spanish population of wormwood from Teruel was introduced in a domestication program (Burillo, 2009, Martín et al., 2011, Martín et al., 2012, Gonzalez-Coloma et al., 2012 and Bailen et al., 2013). The essential oils were characterized by the presence of *cis*-epoxyocimene, chrysanthenol, and chrysanthenyl acetate. These oils repelled reduviid insects and showed moderate effects against fungal pathogens (Sainz et al., 2012 and Bailen et al., 2013). Furthermore, supercritical fluid extracts of this *A. absinthium* population showed an improvement in the yield of several mono- and sesquiterpenes and were more antifeedant than the traditional extracts (hydrodistilled and organic) against phytophagous insects (Martín et al., 2011 and Martín et al., 2012).

In this work, selected germplasm from the above mentioned pre-domesticated *A. absinthium* population (Teruel) entered a second field cultivation cycle for agronomic stabilization and further valorization. Additionally, wild germplasm from another *A. absinthium* population originally from Sierra Nevada (Spain) entered the same cultivation process for pre-domestication and comparison purposes. Here we report on the biomass and essential production of these two populations of *A. absinthium* (Teruel and Sierra Nevada) with different domestication levels and the chemical composition of their essential oils (Clevenger distilled, HDs; and industrial vapor-pressure, VPs) over 6 years of the cultivation process. The insect antifeedant (*Leptinotarsa decemlineata*, *Spodoptera littoralis*, *Myzus persicae* and *Rhopalosiphum padi*) and antifungal (*Fusarium* spp., *Boytrytis cinerea*) actions of these extracts were also tested, being the antifungal effect of the VPs the most important one. The antifungal compounds present in the VP extracts have been identified.

2. Material and methods

2.1. General experimental procedures

Optical rotations were determined in $CHCl_3$ at room temperature using a Perkin-Elmer 137 polarimeter. NMR spectra were measured on a Bruker AMX2 500 MHz spectrometer with pulsed field gradient using the solvent as internal standard ($CDCl_3$, at δ_H 7.26 and δ_C77.0). The programs used in two-dimensional (2D) NMR experiments (HMBC, HSQC, COSY, and NOESY) were those furnished with the manufacturer's software. EIMS and exact mass measurements were recorded on a Micromass Autospec instrument at 70 eV. Preparative flash chromatography was carried out on 2.5 cm diameter silica cartridges (40–70 μm) in a Jones Flash Chromatography apparatus. Silica gel 40–70 μm (Merck) was used for column chromatography. Compounds were visualized on TLC with Oleum reagent.

2.2. Plant material and cultivation

Pre-domesticated plant material (Teruel population): the seeds originated from thujone-free plants submitted to a previous domestication period of 6 years and individually selected based on their biomass and HD essential oil yield at the end of the cycle (T1); (Burillo, 2009 and Gonzalez-Coloma et al., 2012) to give the domesticated T2 population.
Wild plant material (Sierra Nevada population): the seeds were collected from individuals grown in a greenhouse and originated from a population donated by the Sierra Nevada National Park (Granada) (SN0) (Gonzalez-Coloma et al., 2012) to give the pre-domesticated SN1 population.
The seedlings were planted in 2008 in an experimental field located in Ejea de los Caballeros, Zaragoza, Spain. A detailed description of the field has been reported (Burillo, 2009). Along with the cultivated plants, 40 individuals from each parent population (T1 and SN0) were maintained in a separated plot (1 row/parent population) of the same field for comparison purposes. Briefly, 6 randomized blocks with 6 rows/block and 30 individuals/row were planted for each population. Flowering plants were harvested yearly between 2008 and 2013 and processed for agronomic evaluation and extraction. For HD essential oils, each block was extracted individually by pooling 20 plants collected from the central rows (3 and 4, 10 plants from each row) and only block 2 (BII) oils were used for chemical analysis and bioassays. For semi-industrial vapor pressure extraction (VP), plants harvested from all the blocks of each population were pooled (1080 plants/population) and extracted.

2.3. Extraction

Laboratory scale hydrodistillation (HD extracts) was performed in a Clevenger-type apparatus (0.43–0.53% yield) according to the method recommended by European Pharmacopoeia (http://www.edqm.eu/en/Homepage-628.html). Semi-industrial vapor pressure extraction (VP extracts) was carried out in a stainless steel industrial distillation plant (http://www.ecoaromuz.com) equipped with two 3000 l vessels (yield, plant fresh weight/population/year is shown in Table 1).

2.4. GC-MS analysis of essential oils (HD and VP extracts)

The essential oils extracted were analyzed by GC–MS using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5973N mass detector (electron ionization, 70 eV) (Agilent Technologies, Palo Alto, CA, USA) and equipped with a 25 m × 0.20 mm i.d. capillary column (0.2 μm film thickness) HP-1 (methyl silicone bonded) (Hewlett-Packard). Working conditions were as follows: split ratio (20:1), injector temperature, 260 °C; temperature of the transfer line connected to the mass spectrometer, 280 °C; initial column temperature 70 °C, then heated to 270 °C at 4 °C min^{-1}. EI mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001). Quantitative data were obtained from the TIC peak areas without the use of response factors. Values are expressed as average (minimum–maximum range) of individual samples from 3 years (2008 to 2010).

2.5. Compound isolation

A VP extract of *A. absinthium* (20 g, T2 population, 2011) was submitted to vacuum liquid chromatography on a Si-gel column (40–70 μm, 6 cm diameter, 9 cm length) eluted with a hexane (Hx): dichloromethane (DCM) gradient of increasing polarity (0.5–100% DCM). Nine fractions were obtained and analyzed by GC–MS as described. Fraction 8 (690 mg) was further purified by flash chromatography on a 2.5 cm diameter silica cartridge (40–70 μm) eluted with a Hx:DCM (70:30) mixture (isocratic, 18 ml/min flow rate) and by Sephadex LH-20 chromatography (DCM:MeOH, 1:1) to give (−)-*cis*-chrysanthenol (**1**) (33.5 mg; 4.9%).

2.6. Insect bioassays

S. littoralis, *L. decemlineata*, *M. persicae* and *R. padi* colonies were reared on artificial diet, potato (*Solanum tuberosum*), bell pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) plants, respectively, and maintained at 22 ± 1 °C, >70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber. The bioassays were conducted with newly emerged *S. littoralis* L6 larvae or *L. decemlineata*/*M. persicae*/*R. padi* adults as described (Burgueño-Tapia et al., 2008).

2.7. Antifungal bioassays

Fusarium moniliforme (Sheldon) [CECT2152], *F. oxysporum* fs. *lycopersici* (Scheldt) [CECT2715], *F. solani* (Mart.) [CECT2199] were provided by the *Colección Española de Cultivos Tipo* (CET) and *B. cinerea* (Pers.) [B05.10] was donated by the Biochemistry and Molecular Biology Department (ULL, Spain). Antifungal activity was analyzed as mycelial growth inhibition by a modified agar-dilution method which includes the addition of 0.05 mg/ml of methyltetrazolium salts (MTT). Colonies grown on Petri dishes incubated for 48 h were digitalized and measured with the application ImageJ (Image J, http://rsb.info.nih.gov/ij). Percent inhibition (%I) was calculated as: $\%I = (C - T/C) \times 100$, where C is the diameter of the control colonies and T is the diameter of the test colonies. The active compounds were tested

in a dose-response experiment to calculate their relative potency (EC_{50} values, the effective dose to give 50% inhibition).

2.8. Phytotoxic activity

The experiments were conducted with *Lactuca sativa* cv. Teresa (Fito, España), and *Lolium perenne* (Batlle) seeds, as described (Martín et al., 2011). Briefly, 2.5 cm diameter filter paper with 20 µl of the test compound (10 µg/µl for extracts and 5 µg/µl for pure compounds) were placed on 12-well plates (Falcon). Five hundred µl H_2O/well and 10/5 seeds (*L. sativa*/*L. perenne* presoaked in distilled water for 12 h) were added and the covered plates placed in a plant growth chamber (25 °C, 70% RH, 16:8 L:D). Germination was monitored for 6 days and the root/leaf length measured at the end of the experiment (25 plantlets randomly selected for each experiment and digitalized with the application ImageJ 1.43, http://imagej.nih.gov/ij/). A non-parametric analysis of variance (ANOVA) was performed on radical length data.

2.9. Statistical analysis

Multivariate statistical data analysis was carried out using Statgraphics plus 4.1 program package. The variability of the chemical composition of each oil type (HD, VP) was assessed based on relative concentration data (% composition values for each year between 2008 and 2013) subjected to cluster analysis (Nearest Neighbor Method, Squared Euclidean).

3. Results and discussion

3.1. Biomass and essential oil yield

Table 1 shows the biomass and essential oil yields (VPs) of two cultivated *A. absinthium* populations with two domestication levels. The domesticated T2 population yielded higher biomass and essential oil (VP) for all the years collected according to its domestication level.

Table 1. Biomass (fresh weight, kg/ha) and essential oil (EO) yield (l/ha) of two populations of cultivated *A. absinthium*. T2, domesticated Teruel population; SN1, pre-domesticated Sierra Nevada population.

Year	Population			
	Teruel (T2)		Sierra Nevada (SN1)	
	Biomass	EO	Biomass	EO
2008	21494	37.51	18104	30.77
2009	23530	28.00	20621	18.07
2010	21355	33.79	18717	21.34
2011	25446	47.66	23141	27.82
2012	18707	55.45	17402	37.41
2013	21674	31.53	23764	31.98

3.2. Chemical composition of essential oils (HD and VP extracts)

Table 2 shows the average (range) composition of the hydro-distilled extracts (HD) from 3 years (2008 to 2010), analyzed individually. Qualitative and quantitative variations were observed between the two populations. Among the most variable compounds were*cis*-epoxyocimene (**5**), with higher variation for the T population; chrysanthenyl acetate (**7**); (*5Z*)-2,6-dimethylocta-5,7-diene-2,3-diol (**4**) with higher levels in the T samples. Camphor (**3**) and cineol (**6**) correlated with the domestication level of the population. Among the less variable compounds were (−)-*cis*-chrysanthenol (**1**), with higher levels in the SN population; *trans*-caryophyllene; linalool (**2**) and an unknown sesquiterpene alcohol (Fig. 1).

Table 3 shows the average (range) composition of the semi-industrial VP extracts from 6 years (2008 to 2013), analyzed individually. Similarly, qualitative and quantitative differences were also observed. As previously shown for the HDs, the presence of cineol (**6**) and camphor (**3**) correlated with the domestication level of the population. (−)-*cis*-Chrysanthenol (**1**) showed 1.5 times higher levels in the SN1 population while chrysanthenyl acetate (**7**) was 22 times higher in T2. Compound **4** was absent in all the VP extracts probably due to the extraction method.

Table 2. Chemical composition of essential oils (Clevenger hydrodistilled, HDs) of the different *A. absinthium*samples (abundance: % area). Values are expressed as average (range) of individual samples from 3 years (2008 to 2010). T, Teruel population; SN, Sierra Nevada population; T2, domesticated T population; T1, parent T population; SN1, pre-domesticated SN population; SN0, parent SN population.

Compounds	Teruel (2008-2010)		Sierra Nevada (2008-2010)	
	T2	T1	SN1	SN0
1,8-Cineol (**6**)	2.1 (1.3 - 3.6)	0.6 (0.0 - 1.2)	0.3 (0.0 - 0.8)	0 (0.0 - 0.0)
Linalool (**2**)	5.8 (5.5 - 6.1)	2.8 (1.3 - 4.2)	3.1 (2.7 - 3.3)	3.5 (2.3 - 4.4)
(-)-(*Z*)-Epoxyocimene (**5**)	23.4 (0.0 - 36.4)	25.7 (0.0 - 45.9)	35.7 (25.3 - 44.2)	39 (19.7 - 50.9)
Camphor (**3**)	4.2 (3.2 - 4.7)	0 (0.0 - 0.0)	0 (0.0 - 0.0)	0 (0.0 - 0.0)
(-)-*cis*-Chrysanthenol (**1**)	19.5 (18.4 - 21.6)	16 (10.4 - 23.0)	22.9 (13.2 - 28.9)	26.6 (19.8 - 37.7)
Chrysanthenyl acetate (**7**)	2.9 (0.0 - 4.9)	12.3 (0.5 - 20.4)	6.8 (0.0 - 20.3)	0.4 (0.0 - 1.1)
(*Z*)-2,6Dimetillocta-5,7-diene-2,3-diol (**4**)	22 (9.5 - 42.5)	25.9 (0.0 - 53.3)	11.2 (0.0 - 20.8)	3.6 (0.0 - 7.2)
Trans-caryophyllene	1.9 (1.3 - 2.3)	1.6 (1.6 - 1.7)	2 (1.7 - 2.4)	2.1 (0.9 - 3.2)
Sesquiterpene alcohol	1.7 (0.9 - 2.4)	2.2 (1.7 - 2.5)	1.9 (1.8 - 2.1)	1.9 (1.7 - 2.2)

Table 3. Chemical composition of essential oils (semi-industrial vapor pressure extracts, VP) of the different *A. absinthium* samples (abundance: % area). Values are expressed as average (range) of individual samples from 6 years (2008 to 2010). T2, domesticated Teruel population; SN1, pre-domesticated Sierra Nevada population.

Compounds	VP (2008-2013)	
	T2	SN1
α-Pinene	2.1 (1.5 - 2.7)	1.4 (1.0 - 1.7)
1,8-Cineol (**6**)	2.1 (1.7 - 2.4)	0.1 (0 - 0.3)
Linalool (**2**)	3.8 (2.1 - 5.1)	3.2 (1.5 - 4.0)
(-)-(*Z*)-Epoxyocimene (**5**)	43.0 (38.9 - 44.1)	44.6 (37.3 - 49.0)
(*E*)-Epoxyocimene	1.4 (0 - 3.0)	2.3 (1.8 - 3.1)
Camphor (**3**)	4.7 (2.8 - 7.7)	0.2 (0 - 1.1)
(-)-*cis*-Chrysanthenol (**1**)	11.9 (10.4 - 12.7)	18.1 (15.9 - 21.9)
Chrysanthenyl acetate (**7**)	6.6 (5.5 - 7.4)	0.3 (0 - 0.7)
Trans-caryophyllene	4.0 (3.4 - 4.2)	5.2 (4.3 - 5.6)
γ-Cadinene	1.8 (1.3 - 2.8)	2.8 (1.7 - 4.1)

Overall, there was lower chemical variation for the VPs than for the HDs due to the sample size (pooled plants from three experimental blocks, 6 years analyzed) and the extraction method. A dendrogram based on the composition of the HDs (Fig. 2) showed five groups: T2(08–10), T1(09)–SN1(09), T1(10)–SN1(08–10)–SN0(08-10), SN0(09) and T1(10). These groups indicated a larger variation for the parent (T1 and SN0) and the pre-domesticated SN1 populations than the domesticated T2.

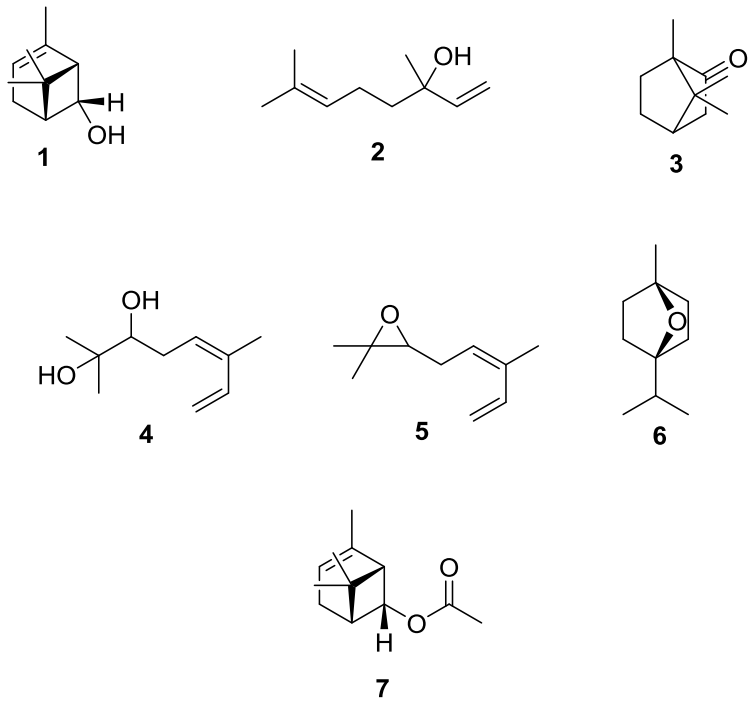

Figure 1. Chemical structures of compounds **1-7**

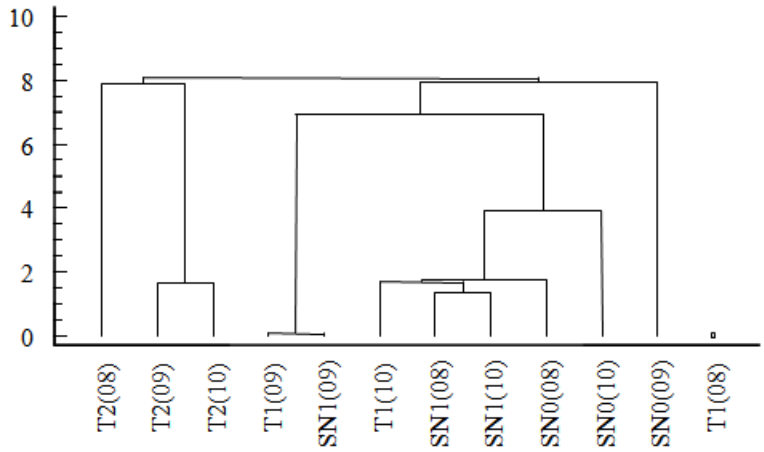

Fig. 2. Dendrogram (Nearest Neighbor Method, Squared Euclidean) generated from cluster analysis of GC–MS data of hydrodistilled extracts (HD) from two populations of cultivated *A. absinthium.* T, Teruel population; SN, Sierra Nevada population; T2, domesticated T population; T1, parent T population; SN1, pre-domesticated SN population; SN0, parent SN population; in parenthesis the crop date (year).

A dendrogram based on the composition of the VPs (Fig. 3) showed two major groups: T2 (except T2(10)) with high levels of compound **4** probably due to an uncontrolled variation in the semi-industrial extraction process, data not shown) and SN1, with higher variation for this second cluster. Therefore, the domestication of the T2 population resulted in lower chemical variation of the essential oils (HDs and VPs), allowing for the registration of a new *A. absinthium* variety (®Candial).

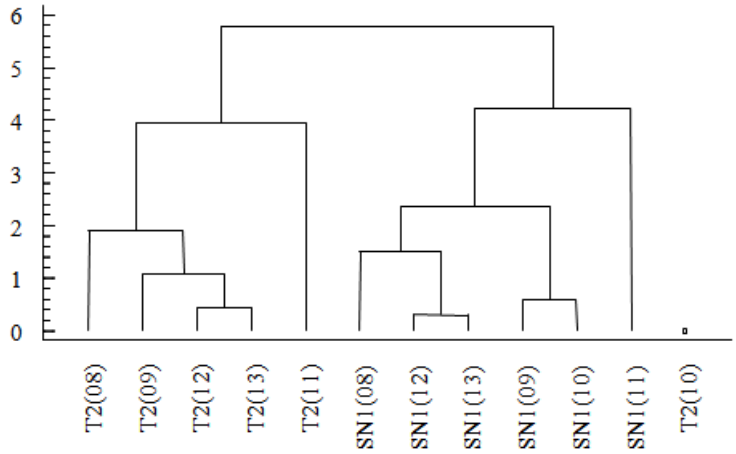

Fig. 3. Dendrogram (Nearest Neighbor Method, Squared Euclidean) generated from cluster analysis of GC–MS data of semi-industrial vapor-pressure extracts (VP) from two populations of cultivated *A. absinthium*. T2, domesticated Teruel population; SN1, pre-domesticated Sierra Nevada population; in parenthesis the crop date (year).

Two similar *A. absinthium* chemotypes have been described from the Iberian Peninsula (Ariño et al., 1999); *cis*-epoxyocimene type and a *cis*-epoxyocimene + chrysanthenyl acetate type. The HDs of the domesticated T2 population showed *cis*-epoxyocimene + *cis*-chrysanthenol chemotype, similar to the T1 parent population (Bailen et al., 2013) but also contained

chrysanthenyl acetate. However, the SN1 population showed a *cis*-epoxyocimene + chrysanthenyl acetate type, suggesting a high phenotypic plasticity for the production of these compounds.

3.3. Antifeedant effects

Table 4 shows the average (range) insect antifeedant effects of essential oil extracts from 3 years (2008 to 2010) tested individually. Overall, the antifeedant effects of the *A. absinthium* essential oils (HD and VP) were moderate-low. *L. decemlineata* was affected by cultivated (T2-VP) and wild (SN0-HD) extracts. *S. littoralis* was affected by cultivated T2 and SN1 HD extracts. The aphids (*M. persicae* and *R. padi*) were not affected. Similarly, previous studies on the antifeedant effects of HDs from the parent germplasm of both populations studied here (T1, SN0) showed no effects on *M. persicae* and low-moderate on *S. littoralis* (Bailen et al., 2013) while their superctitical extracts showed strong antifeedant and insecticidal effects against *S. littoralis*, being the aphids less sensitive (Martín et al., 2011 and Martín et al., 2012), indicating that for insect control purposes supercritical CO_2 extraction should be used.

Table 4. Antifeedant effects of two populations of cultivated *A. absinthium* (Teruel and Sierra Nevada) extracts (HD, hydrodistilled; VP, semi-industrial vapor-pressure extracts) on the target insect species. Values are expressed as average (range) of individual samples from 3 years (2008 to 2010) tested at 100 μg/cm^2.

Germplasm	Extract	Teruel				Sierra Nevada			
		L. decemlineata	*S. littoralis*	*M. persicae*	*R. padi*	*L. decemlineata*	*S. littoralis*	*M. persicae*	*R. padi*
		%FI[a] (range)		%SI[a] (range)		%FI[a] (range)		%SI[a] (range)	
Parent	HD	58.4 (57.5-59.7)	52.9 (40.1-64.6)	49.1 (33-72.9)	47.3 (45.4-50.9)	82.1 (75.9-86.6)	47.8 (40.8-53.2)	53.7 (33.2-66.6)	49.1 (38.1-58.3)
Cultivated	HD	56.9 (54.7-59.5)	66.3 (54.9-74.2)	40.8 (25.7-50.4)	30.0 (27.7-32.4)	52.5 (47-62.3)	69.3 (65.9-71.1)	39.2 (37.5-40.9)	32.8 (25.5-46.7)
	VP	73.2 (63.5-86.3)	54.2 (43.3-62.8)	51.7 (47.6-54.2)	18.4 (8.4-23.6)	43.7 (37.2-52.2)	56.3 (45.9-65.1)	45.6 (38.3-51.7)	21.2 (8.5-37.7)

a Percent feeding (FI)/setting (SI) inhibition.

3.4. Phytotoxic activity

The essential oils from both cultivated *A. absinthium* populations from 3 years (2008 to 2010) were tested for phytotoxic effects against *L. sativa* and *L. perenne*. The germination of *L. sativa* (>99%) and *L. perenne* (>85%) was not affected at the end of the experiments. Overall the growth of *L. perenne* (root: 65–91%, leaf: 50–108%) was more affected by the oils than *L. sativa* (root: 71–150%) (data not shown). Similarly, previous phytotoxicity tests of HDs from the parent germplasm of both populations (T1, SN0) studied here showed no effects on *L. sativa* and *L. perenne* (Bailen et al., 2013).

3.5. Antifungal effects

Table 5 shows the average (range) antifungal effects of essential oil extracts from 3 years (2008 to 2010) tested individually. The semi-industrial vapor pressure extracts (VP) showed stronger antifungal effects that the HDs against all the *Fusarium* species, with the SN1 VP extracts being more effective against *F. moniliforme* and *F. solani* than the T2 ones. These extracts were further tested against *B. cinerea*, being this species the most sensitive one. Previous studies on the antifungal effects of HDs from both parent populations (T1, SN0) showed moderate action against *Fusarium* spp. (Bailen et al., 2013), being these effects lower that the ones studied here (VPs). Therefore, for antifungal applications vapor-pressure extraction should be used.

Table 5. Antifungal effects (mycelial growth inhibition) of two populations of cultivated *A. absinthium* (Teruel and Sierra Nevada) extracts (HD, hydrodistilled; VP, semi-industrial vapor-pressure) on the target fungal species. Values are expressed as average (range) individual samples from 3 years (2008 to 2010) tested at 1 mg/ml.

Germplasm	Extract	Teruel				Sierra Nevada			
		F. moniliforme	*F. oxysporum*	*F. solani*	*B. cinerea*	*F. moniliforme*	*F. oxysporum*	*F. solani*	*B. cinerea*
Parent	HD	31.0	42.2	34.0	nt	34.2	45.0	59.7	nt
		(25.1 – 37.0)	(38.0-46.5)	(19.2-48.8)	nt	(19.9-48.4)	(27.7-62.0)	(49.8-69.5)	nt
Cultivated	HD	36.7	51.1	54.2	nt	36.8	56.8	42.2	nt
		(28.1- 45.2)	(45.5-56.7)	(37.2-71.1)	nt	(31.1-42.5)	(48.9-64.6)	(19.6-64.8)	nt
	VP	100	100	100	100	100	100	100	100
	ED_{50}	(0.24-0.43)	(0.29-0.40)	(0.24-0.50)	(0.01-0.07)	(0.11-0.24)	(0.20-0.50)	(0.11-0.27)	(0.01-0.03)

a ED_{50}, concentration needed to produce 50% mycelial growth inhibition (mg/ml).

3.6. Compound isolation and characterization

The fractionation of a VP extract resulted in the identification of two active fractions (7 and 8, Table 6). Fraction 7 was rich in (−)-*cis*-chrysanthenol (**1**), camphor (**3**) and linalool (**2**) with small amounts of (−)-*cis*-epoxyocimene (**7**) and traces of hexenyl butyrate. Fraction 8 was also rich in **1** followed by **2** and hexenyl butyrate (Table 7).

Table 6. Antifungal effects (mycelial growth inhibition) of fractions 1–9 from T2 (11) VP extract and compounds **1**–**3**on the target fungal species.

	Sample	Concentration (mg/ml)	*F. oxysporum*	*B. cinerea*
Fraction	1	1.0	31.19 ± 2.6	46.62 ± 1.4
	3	1.0	54.62 ± 2.1	46 ± 1.0
	4	1.0	49.12 ± 3.1	47.03 ± 1.9
	5	1.0	51.07 ± 1.8	41.09 ± 1.4
	6	1.0	45.09 ± 4.2	35.13 ± 1.2
		1.0	100 ± 0.0	100 ± 0.0
	7	0.5	68.00 ± 1.8	45.37 ± 2.2
		0.1	-34.54 ± 5.5	-19.46 ± 2.3
	9	1.0	52.58 ± 4.5	29.28 ± 2.0
		1.0	100 ± 0.0	100 ± 0.0
Compound	(-)-*cis*-Chrysanthenol (**1**)	0.5	68.3 ± 1.5	na*
		0.1	na	na
	Linalool (**2**)	1.0	70.38±2.1	100±0.0
		0.5	45.54±2.5	na
		0.1	7.58 ± 3.7	na
	Camphor (**3**)	1.0	7.6 ± 2.3	21.6±3.7

*na, not available.

Table 7. Chemical composition of T2(11) VP extract and fractions 1–9 (abundance: % area).

Compound	VP	Fraction							
	T2(11)	VLC1	VLC3	VLC4	VLC5	VLC6	VLC7	VLC8	VLC9
Yield (%)	-	12.6	0.8	7.6	5.3	13.5	4.8	7.8	1.7
Linalool (**2**)	2.0	-	-	-	-	-	-	17.3	-
(-)-(*Z*)-Epoxyocimene (**5**)	39.8	-	-	13.5	63.9	86.6	5.6	-	-
Camphor (**3**)	4.5	-	-	-	1.4	7.2	27.0	-	-
(-)-*cis*-Chrysanthenol (**1**)	11.9	-	-	-	-	-	60.8	63.0	0.7
(*E*)-3-hexenyl butyrate	1.1	-	-	-	-	-	1.7	12.4	-
Chrysanthenyl acetate (**7**)	5.3	-	1.0	35.7	22.1	0.9	-	-	-
(5*Z*)-2,6-dimethylocta-5,7-diene-2,3-diol (**4**)	2.0	-	-	-	-	-	-	-	67.8
trans-Caryophyllene	3.8	29.5	-	-	-	-	-	-	-
Germacrene-D	2.3	15.5	-	-	-	-	-	-	-
β-Selinene	1.1	8.8	-	-	-	-	-	-	-
Dihydrochamazulene	5.8	2.5	7.9	-	-	-	-	-	-
Chamazulene	2.6	1.1	81.7	-	-	-	-	-	-

Compounds **1**–**3**, present in the active fractions were tested individually. **1** and **2** were antifungal but not **3** (Table 7). Compound **7**, also present in the active fractions and not available, was most likely inactive since fraction 6, rich in **7** (87%), was not antifungal. Therefore, (−)-*cis*-chrysanthenol (**1**) was the main antifungal agent of the VP extracts, followed by linalool (**2**), present in lower amounts. The small difference in antifungal effects between the T2 and SN1 VP extracts could be explained by their different content in **1** (Table 3).

The HDs were inactive or showed moderate activity (Table 6) against *Fusarium* spp., being their content in the inactive (*5Z*)-2,6-dimethylocta-5,7-diene-2,3-diol (**4**) (see fraction 9, Table 7), their main chemical difference (higher content in HDs) respect to the VPs (Table 2 and Table 3).

(−)-*cis*-Chrysanthenol (**1**) has not been previously reported as being antifungal. Linalool (**2**) is a known antimicrobial and antifungal agent involved in plat defense against pathogens (Kuorwel et al., 2014, Shimada et al., 2014 and Tanaka et al., 2014). (*1R*)-(+)-Camphor inhibited the mycelial growth of *Choanephora cucurbitarum*, a plant pathogen (Pragadheesh et al., 2013) but resulted inactive against the fungal species tested here.

4. Conclusions

The domestication of an *A. absinthium* population from Teruel (Spain) resulted in lower chemical variation and higher biomass and essential oil yields, allowing for the registry of a new domesticated variety (®Candial). The main components of its essential oil extracts were *cis*-epoxyocimene, chrysanthenol, chrysanthenyl acetate, *trans*-caryophyllene and linalool. (*5Z*)-2,6-Dimethylocta-5,7-diene-2,3-diol was only present in the hydro-distilled HD extracts. The presence of cineol and camphor correlated with the domestication level of *A. absinthium*. The semi-industrial vapor-pressure extracts (VPs) showed strong antifungal effects. (−)-*cis*-Chrysanthenol (**1**) has been identified as the main antifungal agent present in the VP extracts, followed by linalool (**2**). Therefore, compound **1** should be used as the chemical indicator of the antifungal quality of VP extracts from *A. absinthium* (®Candial).

Acknowledgments

This work has been supported by grants CTQ2012-38219-C03-01 (Spain) and JAE-CSIC (predoctoral fellowship to L.F. Julio).

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4.3. Nematicidal activity of hydrolate from *Artemisia absinthium* var. ®candial

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Manuscript in preparation **a**

Abstract

Hydrolate obtained as a by-product of the essential oil extracted from a domesticated population of *Artemisia absinthium* were tested against the root knot nematode *Meloidogyne javanica*. The hydrolate showed strong in vitro nematicidal effect up to a dose of 50%. The hydrolate strongly suppressed *M. javanica* egg hatching (> 95%) after 5 days of incubation up to a 50% dose. The *in vivo* tests on tomato seedlings showed a significant reduction of J2 infectivity at a sublethal dose (33 %). In pot experiments, the hydrolate of *A. absinthium*, tested on tomato plants significantly reduced the nematode population, infection frequency and reproduction rate. The organic extraction of the hydrolate gave a nematicidal extract, with the aqueous fraction being inactive. This study demonstrates that the organic fraction of the *A. absinthium* hydrolate is a potential root-nematode control agent. Such byproduct of the essential oil extraction therefore represents a sustainable and environmentally friendly alternative to the use of chemical nematicides.

Keywords: root-knot nematode, *Meloidogyne javanica*, *Artemisia absintium,* hydrolate, organic fraction, nematicidal activity.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are major threats to agriculture worldwide (Bird and Bird, 2001). They are obligate endoparasites that infect a large range of crop plants, leading to considerable economic losses of several millions of dollars worldwide infection takes place when the motile second-stage juvenile (J2), is attracted to the root system of the host plant. The infective second-stage juveniles (J2s) migrate intercellularly toward the vascular cylinder after penetrating the root tips, selecting specific parenchyma cells that become their permanent feeding sites (Escobar *et al.*, 2015). During its development, the nematode feeds on host plant nutrients and water. This impairs plant growth, causes wilting, increases the susceptibility of the plant to other pathogens and under some conditions may kill the plant.
Synthetic nematicides have been used to protect crops in intensive productions systems throughout most of the 20th century. In the last decades, environmental and human health concerns have steadily reduced the availability of efficient commercial nematicides (Nyczepir and Thomas, 2009; Sorribas and Ornat, 2011). Therefore, environmentally friendly substances for effective nematode control are needed. In this context, phytochemicals, including essential oils, have a great potential in nematode control (Chitwood, 2002; Andrés *et al.*, 2012).
Artemisia absinthium L. (wormwood) is a perennial plant of the family Asteraceae widely studied. Thujone-free Spanish populations of wormwood have been domesticated for cultivation, resulting in a chemically stable new variety (Burillo, 2009; Martín *et al.*, 2011a; Gonzalez-Coloma *et al.*, 2012; Bailen *et al.*, 2013; Julio *et al.*, 2015). The composition of the essential oils (EOs), ethanolic and CO_2 supercritical fluid extracts of these populations have been described with the EOs characterized by the presence of (-)-cis-epoxyocimene, (-)-cis-chrysanthenol, linalool and chrysanthenyl acetate (Martín *et al.*, 2011a; Julio *et al.*, 2015). These showed insect antifeedant (Martín *et al.*, 2011a), antifungal (Kuorwel *et al.*, 2014; Julio *et al.*, 2015), antiparasitic activity (Gonzalez-Coloma *et al.*, 2012; Martínez-Díaz *et al.*, 2015) and nematicidal effects against animal parasitic nematodes (Vegas-Sanchez *et al.*, 2015).
The pilot plant vapor pressure extraction of the EO from the domesticated variety of *A. absinthium* generates a hydrolate. In this work we have demonstrated the *in vitro* and *in vivo* nematicidal activity of this hydrolate against *M. javanica*. Furthermore, the activity remained in its organic fraction.

2. Materials and methods

2.1 Plant material

A detailed description of the field and the cultivation parameters has been reported (Burillo, 2009). Flowering plants were harvested yearly and processed for extraction as described (Julio *et al.*, 2015). The hydrolates studied here corresponded to crops collected between 2008-2010 from two experimentally cultivated populations (T and SN).

2.2 Extraction and fractionation

Semi-industrial vapor pressure extraction (VP extracts) was carried out in a stainless steel industrial distillation plant (http://www.ecoaromuz.com) equipped with two 3,000 L vessels (Julio et al., 2015). The hydrolate was collected after the essential oil was decanted and filtered. The hydrolate was extracted by solid phase extraction using 100 mL of residual water and 10 g of activated carbon (activated charcoal granulate, Scharlau) to give an oily organic extract (0.45% yield).

2.3 Nematicidal activity evaluation

A field- selected *M. javanica* population from Barcelona (Spain) was maintained on *Solanum lycopersicum* L. plants (var. Marmande) in pot cultures at 25 ± 1 °C, >70% relative humidity. Egg masses of *M. javanica* were handpicked from infected tomato roots two months after inoculation of the seedlings. Second-stage juveniles (J2) were obtained by incubating egg masses in a water suspension at 25 °C for 24 hours. Other egg-masses were dissolved with 0.5 % sodium hypochlorite (Hussey and Barker, 1973), and the eggs were collected for pot experiments.

2.3.1 *In vitro* effect on J2

The concentrations of hydrolate tested were 100%, 50%, 33%, 25% and 10%. The initial concentration tested of the organic fraction was 1 mg mL^{-1}. A nematode inoculum (500 J2 in water) was filtered (25 µm) and the nematodes suspended in 500 µL of *A. absinthium* hydrolate solutions. Four aliquots (100 µL) of the nematode suspension (approximately 100 J2) and controls (water) were placed in 96-well plates (BD Falcon, San Jose, CA, USA). The organic extract was dissolved in distilled water containing 5% of a DMSO-Tween solution (0.5% Tween 20 in DMSO) and tested as described (Andrés *et al.*, 2012). The nematicidal activity data are presented as percent dead J2s corrected according to Scheider-Orelli's formula. Effective lethal doses (LC_{50} and LC_{90}) were calculated by Probit Analysis.

2.3.2 *In vitro* effect on egg mass hatching

Three sterilized healthy egg masses of nearly uniform size were transferred to a 96-well plate containing 400 µL of *A. absinthium* hydrolate (T2009). Egg masses placed in sterilized distilled water were used as controls. Each experiment was replicated 4 times. The plates were covered to prevent evaporation and incubated in darkness at 25ºC. After 5 days the hatched J2s were counted and the test solutions were replaced with sterilized distilled water. The egg masses were monitored during 4 weeks, until hatching was complete in the control. Relative hatch suppression rate (compared with the controls) were calculated.

2.3.3 Effect on J2 infection capacity

Tomato seeds (susceptible variety, Marmande) were germinated, incubated in a growth chamber (25 ± 2 ºC, 60% RH, 16:8 L:D) for three weeks and transplanted into 5-cm diameter clay pots filled with 10 ml of quartz sand. The seedlings were individually inoculated with 180-

200 J2 untreated (control) or treated with *A. absinthium* hydrolate (T2009) at a sublethal dose of 33% and incubated for one week at the same environmental conditions. The seedlings were then removed from the pots and the roots stained with acid fuchsine (Byrd *et al.*, 1983). Juveniles within the roots of each individual seedling were counted by examining the entire root system under a stereomicroscope. The experiment consisted of six replicas and was repeated three times. Relative percentages of J2 penetration (compared with the controls) were calculated to obtain inhibition rates of J2 infectivity.

2.3.4 Effect on *M. javanica* reproduction in tomato plants

Two concentrations (60% and 20%) of *A. absinthium* hydrolate (T2009) were tested in pot experiments according Oka et al (2014). The solution (10 mL) was mixed with 1.200 kg of sandy loam soil, transferred into 1000 ml plastic pots and moistened with water (15% w/w). Each pot was inoculated with 2000 *M. javanica* J2 and incubated for five days in a growth chamber (25 ± 2 °C, 60% RH, 16:8 L:D). Untreated soil was used as control. After the incubation period, one-month-old tomato (cv. Marmande) seedlings were transplanted, maintained under the same conditions, and fertilized with 50 mL of a 0,3% solution of 20-20-20 (N-P-K) every ten days. After 60 days, at harvest, roots plants were processed according to (Verdejo-Lucas *et al.*, 2012). Nematode infectivity was estimated by counting the number of egg masses per plant (EMs). The infection frequency of the nematode was calculated dividing the number of EMs per plant by the number of eggs inoculated per pot. Egg production was determined by extracting the eggs from the entire root system (Hussey and Barker, 1973). The fecundity of females was estimated as the number of eggs per plant divided by the EMs per plant. The multiplication rate of the nematode (Pf/Pi) was determined dividing the number of eggs per plant (Pf) by the egg inoculum (Pi). Data were transformed [log10 (x+1)] before analysis of variance, and means separated by LSD at $P< 0.05$.

2.4 Phytotoxic activity

The experiments were conducted with *Lactuca sativa cv* Teresa (Fito, España) and *L. perenne* seeds as described (Martín *et al.*, 2011) Germination was monitored for 6 days and the rootlet length measured at the end of the experiment (25 plantlets randomly selected for each experiment, digitalized and measured with the application Image J, http//rsb.info.nih.gov./ij/). A non parametric analysis of variance (ANOVA) was performed on radical length data.

3. Results and Discussion

The nematicidal activity of hydrolates obtained as subproducts of the vapor-pressure extraction of two domesticated *A. absinthium* populations (T and SN) collected for three years (2008-2010) have been evaluated *in vitro* against *M. javanica* J2 (Table 1). All hydrolates showed strong nematicidal effects, up to a dose of 50%. These results indicate that the hydrolate toxicity is not influenced either by the population or crop.

The *M. javanica* egg hatchability was strongly reduced by the hydrolate treatment after 5 days of incubation (> 95% reduction for both concentrations tested) (Table 2). This effect slowly decreased with time, but at the end of the experiment (28 days) both hydrolate concentrations inhibited over 75% hatching. These results confirm that J2 are more sensitive to the effects of extracts than egg masses (Andrés *et al.*, 2012). The high hatching inhibition rates obtained demonstrate the ability of the *A. absinthium* hydrolate to penetrate the gelatinous matrix and act on nematode eggs.

Table 1. Nematicidal effect of *A. absinthium* hydrolates (T and SN populations, 2008-2010 crops) on second stage juveniles (J2) mortality of *M. javanica.*

Concentration (%)	T			SN		
	2008	2009	2010	2008	2009	2010
100.0	100.0 ± 0.0[a]	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 1.0	100.0 ± 0.0	100.0 ± 0.0
50.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
33.3	17.2 ± 1.2	10.1 ± 2.9	20.4 ± 3.2	24.1 ± 2.7	11.5 ± 4.2	15.9 ± 0.2
25.0	6.4 ± 3.5	6.2 ± 0.8	18.5 ± 1.6	4.3 ± 2.4	6.2 ± 2.7	3.8 ± 0.6
10.0	1.2 ± 0.5	0.0 ± 0.0	-	0.2 ± 2.4	0.0 ± 0.0	-
LC_{50}	37.3	38.2	35.3	36.3	37.6	38.8
CI_{95}	(36.3-38.3)	(37.1-39.2)	(34.2-36.4)	(35.3-37.4)	(36.6-38.6)	(37.7-40.0)
LC_{90}	48.4	48.1	45.6	47.2	47.1	46.1
CI_{95}	(46.8-50.4)	(46.5-50.2)	(43.9-47.7)	(45.5-49.2)	(45.6-48.9)	(44.5-48.2)

[a]Mortality was observed 72 h after treatment. Corrected according to Scheider–Orelli's formula. Values are means of four replicates. LC: Lethal concentration, CI_{95} = confidence interval of the at 95% probability.

Table 2. Egg hatching inhibition effects of *A. absintium* (T09) hydrolate on *M. javanica* egg masses with time.

Concentration %	Relative hatch suppression rate (%)[a] in time[b]				
	0	**7**	**14**	**21**	**28**
100	96.8	87.5	82.8	81.2	80.5
50	95.4	79.2	82.3	79,58	75.1

[a]Each value represents the hatch inhibition rate in the respective treatment corrected according to the control (Scheider–Orelli's formula). Values are means of four replicates.

[b]time 0: after 5 days of immersion in test solutions; time 7:7 days of immersion in water after time 0; time 14: 14 days of immersion in water after time 0; time 21: 21 days of immersion in water after time 0; time 28: 28 days of immersion in water after time 0.

The *in vivo* tests on tomato seedlings showed a strong suppression of J2 infection capacity when treated with the hydrolate (T2009, 33% concentration) (Fig. 1). The three experiments showed similar effects with a significant decrease of J2 root penetration (78, 71 and 68 % respectively) respect to the control (untreated J2). Therefore, a sublethal hydrolate concentration (10% J2 mortality) strongly suppressed the infective capacity of *M. javanica* nematodes lowering their penetration and root colonization. This is the first description of anextract acting at a sub lethal dose on nematode infectivity.

A. absintium hydrolate treatments to the soil in pot experiments (20 and 60% concentrations) significantly reduced *M. javanica* EMs, infection frequency, eggs/g root and Pf/Pi values respect to the control (Table 3). A hydrolate dose of 60% exhibited the highest activity, causing significant reductions (> 80% and > 50%) of nematode egg production and

multiplication rate. The tomato growth was not influenced by these treatments (data not shown). Although *in vitro* tests are an important step for the selection of plant extracts with nematicidal action against root-knot nematodes, *in vivo* tests are needed to further test these results and rule out unwanted phytotoxic effects.

The organic fraction of the hydrolate obtained from solid phase extraction induced high *in vitro* J2 mortality (91.4%), at 1mg/ml (LC_{50}= 0.941 mg/ml), while the water residue (WR) was inactive, indicating that the active compounds were in the organic fraction.

Table 3. Soil effects of on reproductive traits of *M. javanica* in tomato plants when treated with *A. absintium* hydrolate (T2009).

Concentration		egg masses/plant	eggs /plant x 1000	Infection frecuency[a]	Pf/Pi[b]
%	(OF mg /kg soil)				
Control	Control	152.5 ± 8.2a	58.1 ± 6.4a	0,081 ± 0,01a	29.0 ± 3.2a
20	10	66.0 ± 14.7b	19.8 ± 6.2b	0,033 ± 0,02b	9.9 ± 3.1b
60	30	41.8 ± 7.3c	9.4 ± 1.3c	0,021 ± 0,02c	4.7 ± 0.7c

Values are mean ±standard error of six replicated plants. Values within the same column followed by different lower-case letter are significantly different according to Least Significant Difference (LSD) test (P <0.05).

[a] Egg masses per plant/egg inoculum.

[b] Multiplication rate: Eggs per plant/egg inoculum.

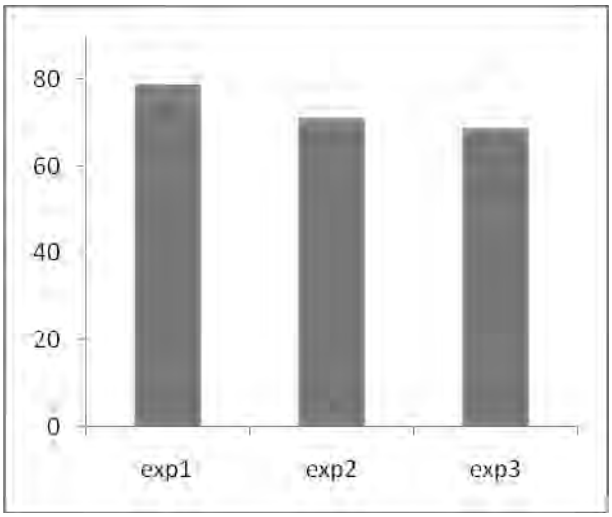

Figure 1. Inhibition rate of penetration of *M. javanica* juveniles (treated with *A. absinthium* hydrolate at a sublethal dose of 33 %) in tomato seedling roots.

This is the first report on the nematicidal activity of an *A. absinthium* hydrolate and its organic fraction. Previous reports have shown that aqueous extracts of *A. absinthium* aerial parts caused *M. incognita* J2 mortality (Dias *et al.*, 2000). However, *A. absinthium* EOs including the populations used here were not nematicidal against *M. javanica* (García-Rodríguez *et al.*, 2015), the pine wood nematode *Bursaphelenchus xylophilus* (Barbosa *et al.*, 2010b) and the stem nematode *Ditylenchus dipsaci* (Zouhar *et al.*, 2009). Hydrolates are by-products of EO extraction processes and contain small amounts of plant volatiles that remain dissolved in the

H_2O phase. Some of these volatile compounds with high polarity do not occur in the essential oils (Wajs-Bonikowska *et al.*, 2015).

Figure 2 shows the phytotoxic effects of the hydrolate (at 100, 50 and 25% solutions) and the organic extract (OE) of *A. absinthium*. The aqueous WR strongly inhibited the germination at the beginning of the experiment (*L. sativa* and *L. perenne*) and growth with stronger effects on *L. perenne*. The organic extract (OE) had no effect on germination at the end of the experiment or plant growth for both species tested.

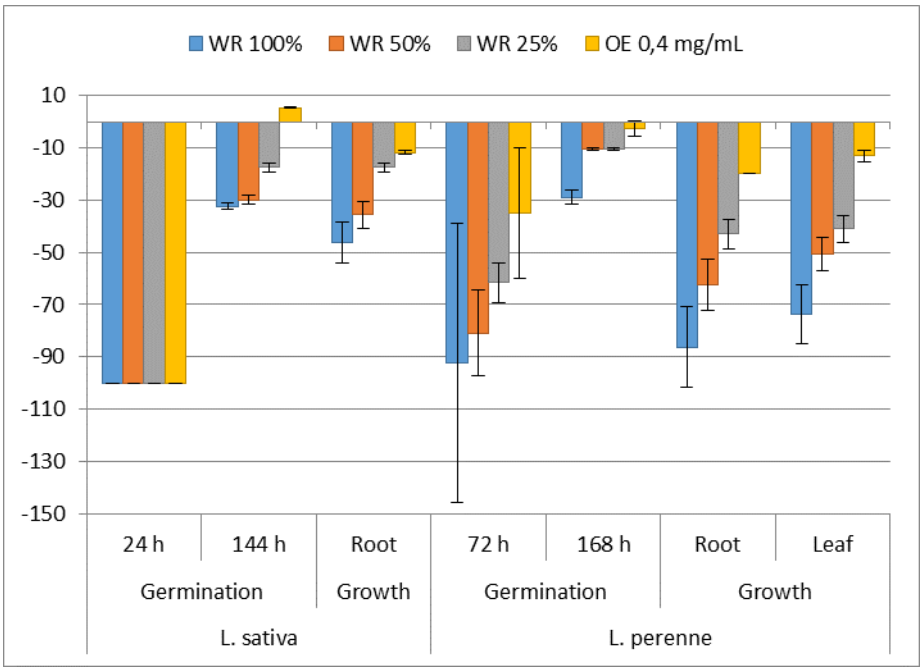

Figure 2. Phytotoxic activity of hydrolate (WR) and organic extract (OE) against *Lactuca sativa and L. perenne* germination and root length (expressed as % of growth inhibition respect to the control).

In conclusion, this study demonstrated, for the first time, the nematicidal activity of an *A. absintium* hydrolate against the root-knot nematode *M. javanica*. Hydrolate treatments caused high mortality of juveniles, inhibited egg hatch, suppressed juveniles infectivity and reduced nematode infection of tomato plants the active compounds were present in the organic fraction of the hydrolate. Therefore, *A. absinthium* hydrolate organic fraction is a potential root-knot nematode control agent. Further research is needed to identify the active compound responsible for the nematicidal effect of the hydrolate organic fraction.

Acknowledgements

This work has been supported by grant CTQ2012-38219-C03-01 (Spain), and JAE-CSIC (predoctoral fellowship to L.F. Julio).

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4.4. Chemical characterization of the nematicidal components of the hydrolate byproduct from *Artemisia absinthium* vapor pressure extraction.

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*Manuscript in preparation **b***

Abstract

The chemical characterization of a nematicidal byproduct (hydrolate) obtained from the extraction of essential oil by vapor-pressure steam distillation of a domesticated population of Spanish *Artemisia absinthium* (var. Candial) led to the isolation of two known monoterpene diols, (Z) and (E)-2,6-dimethylocta-5,7-diene-2,3-diol (**1** and **2**), five tetrahydrofuranoid isomer derivatives (**3**-**7**), four tetrahydropyranoid isomer derivatives (**8**-**11**), and six tetraols (**12**-**17**). Compounds **4**-**6** and **8**-**17** have not been previously described. Among the tetrahydrofuranoids, compounds **3** and **4** were stereoisomers of 5-(2-hydroxypropan-2-yl)-2-methyl-2-vinyltetrahydrofuran-3-ol. Compounds **5** and **6** were stereoisomers of 5-(2-hydroxybut-3-en-2-yl)-2,2-dimethyltetrahydrofuran-3-ol. The four tetrahydropyranoid derivatives (**8**-**11**) were stereoisomers of 2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3,5-diol. Among the more polar tetraols (**12**-**17**), four of them were stereoisomers of 2,6-dimethyloct-7-ene-2,3,5,6-tetraol (mixtures 1:1 of **12**:**13** and **14**:**15**) and two isomers of (*E*)-3,7-dimethyloct-2-ene-1,4,6,7-tetraol (**16** and **17**). Compound **1** showed nematicidal effects against *Meloidogyne javanica*.

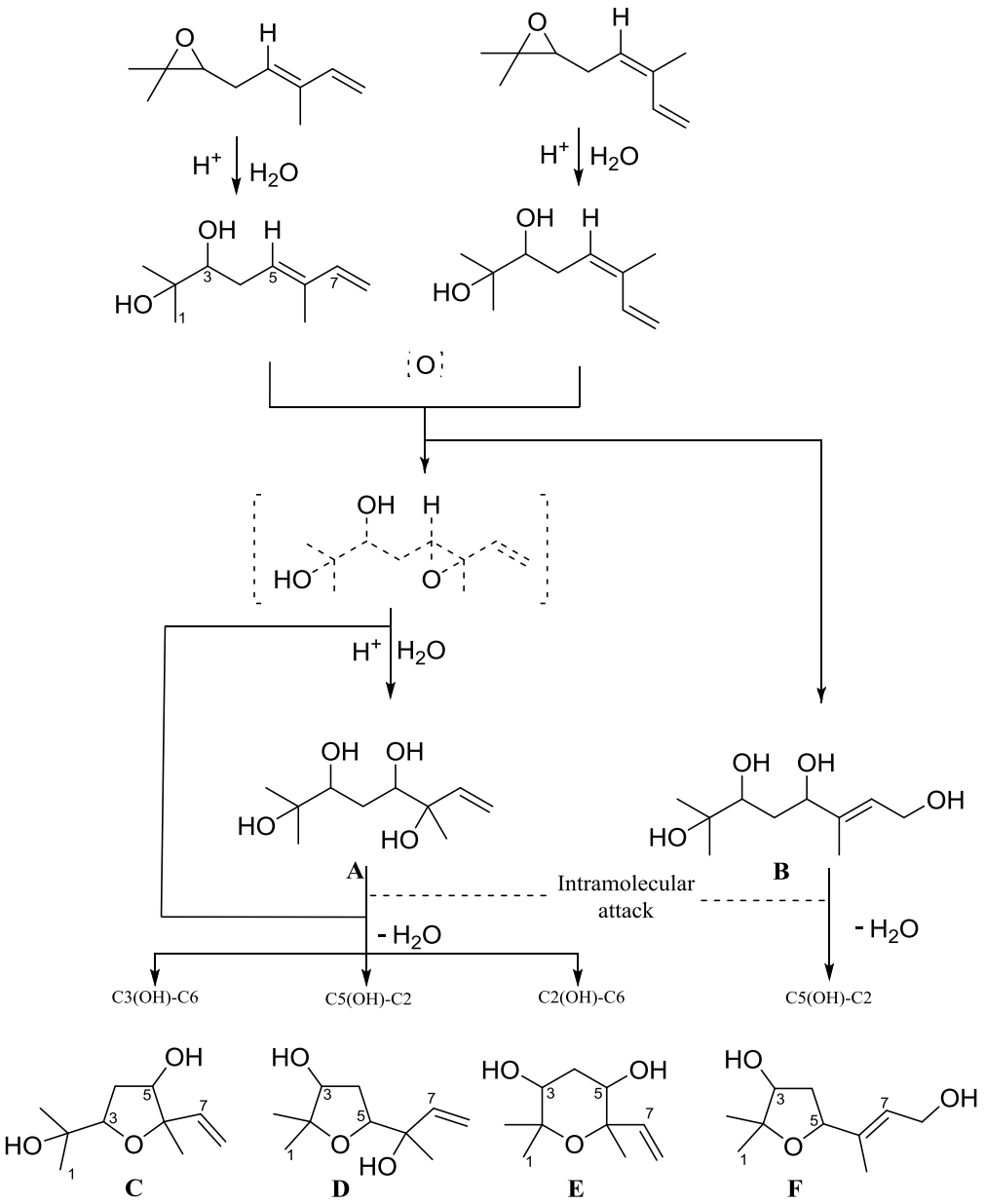
Introduction

Hydrolate is the residual extract obtained as a byproduct in the process of extraction of essential oil by steam distillation. Hydrolates are characterized by the presence of polar volatiles compounds, usually oxygenated (alcohols, oxides, ketones, aldehydes, esters) which in most cases are also present in the chemical composition of the essential oil (Paolini *et al.*, 2008; Maciąg and Kalemba, 2015). The concentration (relative percentage) of volatile oxygenated compounds present in the hydrolate organic extracts is usually higher than in the essential oils (Paolini *et al.*, 2008). The compounds present in the hydrolates could be biosynthetic derivatives or formed in the process of extraction from less polar compounds present in the plant. Hydrolates are used in aromatherapy (Jeannot *et al.*, 2003), in the cosmetic industry (Catty, 2001; Price, 2004;), in food preservation (Tornuk *et al.*, 2011; Sağdıç *et al.*, 2013), as fungicides (Boyraz and Özcan, 2005, 2006) and antioxidants (Ćavar and Maksimović, 2012). Hydrolates can also be a sustainable source of new biopesticides. A recent report showed that a hydrolate from a population of *Lavandula luisieri* under domestication was a strong nematicidal against *Meloidogyne javanica* and showed phytotoxic activity against *Lactuca sativa* and *Loluim perenne* (Julio *et al.,* under revision).

Artemisia absinthium L. is a widely studied perennial plant of the family Asteraceae. Extracts of this plant have been reported as being antifeedant (Martín *et al.*, 2011a), insecticidal (Kordali *et al.*, 2006; Umpiérrez *et al.*, 2012), antimicrobial (Sengul *et al.*, 2011; Baykan Erel *et al.*, 2012), antioxidant (Riahi *et al.*, 2013), antifungal (Kuorwel *et al.*, 2014; Julio *et al.*, 2015) and antiparasitic (Gonzalez-Coloma *et al.*, 2012; Martínez-Díaz *et al.*, 2015).
Thujone-free Spanish populations of wormwood have been domesticated for cultivation (Burillo, 2009; Martín *et al.*, 2011a; Gonzalez-Coloma *et al.*, 2012; Bailen *et al.*, 2013; Julio *et al.*, 2015). The composition of these populations have been described, with the sesquiterpene lactone hydroxypelenolide being the major component, followed by the flavones artemetin and casticin (Gonzalez-Coloma *et al.*, 2012).The EOs were characterized by the presence of (-)-*cis*-epoxyocimene, (-)-*cis*-chrysanthenol, linalool and chrysanthenyl acetate among others, being (-)-*cis*-chrysanthenol the main antifungal agent of the EO (Julio *et al.*, 2015). A recent report on the valorization of the extraction byproducts of this domesticated population of *A. absinthium* showed that the hydrolate was a strong nematicidal against *M. javanica,* with the activity remaining in the organic extract (Julio *et al.*, Manuscript in preparation).
In this work we report on the chemical characterization of the organic extract from a hydrolate obtained as a byproduct of the steam distillation of *A. absinthium*, resulting in the isolation of the known compound **1**-**3**, **7** and the new tetrahydrofuranoids (**4**-**6**), tetrahydropyranoids (**8**-**11**) and tetraols (**12**-**17**). The chemical characterization was carried out using chromatographic techniques, mass spectrometry and nuclear magnetic resonance (NMR 1D and 2D). The nematicidal agent was also identified.

Results and Discussion

Workup of the organic extracts led to the identification of seventeen oxygenated monoterpenes, eleven were diols from the less polar fractions and six were tetraols from the more polar fractions (Fig.1). All these compounds are related to (-)-*cis*-epoxyocimene (Moore *et al.*, 1999) (Scheme 1), the main component of the essential oil (Julio *et al.*, 2015).



Scheme 1. Possible biosynthetic pathway ofcompounds of hydrolate *A. absinthium*

The known monoterpenes diols **1** and **2** were stereoisomers of 2, 6-dimethylocta-5,7-diene-2,3-diol (Tsankova and Bohlmann, 1983; Anisimov, 1992; Fournier-Nguefack *et al.*, 1997; Moore *et al.*, 1999; Osorio *et al.*, 2000; Agrebi *et al.*, 2012). Diol **1** has been previously isolated from an acetone extract of *A absinthium (Bailen et al., 2013)* and was also obtained by acid hydrolysis of epoxyocimene. Among the tetrahydrofuranoids, compound **3** (Osorio *et al.*, 2002) and sachanilol C (**7**) has been previously reported (Fan, 2001; Simon, 2011).

The ^{1}H-NMR data (Table 1) of compounds **3**-**6** and **8**-**15** showed similar spectra, with three signals corresponding to methyl groups (Me-1, Me-9 and Me-10); two methylenes, one aliphatic (H-4β y H-4α) and other olefinic (H-8β y H-8α); three methines, two geminal to oxygen (H-3 and H-5) and one on an olefinic carbon (H-7). The ^{13}C-NMR spectra confirmed the presence of the three methyl groups (C-1, C-9 and C-10); two methylenes, one aliphatic (C-4)

and another vinyl (C-8); three methines, two of them oxygenated (C-3 and C-5) and one olefinic (C-7); as well as two oxygenated quaternary carbons (Table 2).
The COSY experiment (^{1}H-^{1}H) showed correlations between aliphatic methylenes (H-4β and H-4α) and oxygenated methines (H-3 and H-5), and correlations between the protons of the vinyl group (H-7, H-8a and H-8b). A detailed study of the HSQC and HMBC experiments of these compounds showed some significant correlations between the vinyl protons (H-7, H-8a and H-8b) and the carbons C-5, C-6 and C-10; between protons of Me-10 carbon with C-5, C-6, C-7 and C-8 and the protons of Me-1 with C-2, C-3 and C-9. Considering the information described above, compounds **3**-**6** and **8**-**15** have the connectivity shown in fragment **I** (Fig.2).

Compounds **3**-**6** and **8**-**11** showed the same molecular formula $C_{10}H_{18}O_3$, determined by HRESI-TOFMS, indicating that they all had two degree of unsaturations. According to their NMR data each compound had a double bond in its structure (vinyl group), therefore the remaining unsaturation was due to the presence of a ring in the molecule.
The molecular molecular $C_{10}H_{20}O_4$determined by HRESI-TOFMS of compounds **12**-**15** indicated a degree of unsaturation, corresponding to the double bond between C-7 and C-8 observed in the ^{1}H- and ^{13}C-NMR spectra. Therefore, these compounds have a linear structure.

The main difference between compounds **3**-**6** and compounds **8**-**15** was the chemical shift values for the sp^3 hybridization states of the oxygenated carbons (oxymethines and quaternary carbons) of **3**-**6** being higher than 80 ppm. The chemical shifts for the methines of compounds **3**-**6** were 83.2, 83.0, 81.2 and 81.6 respectively (C-3 in the case of compounds **3** and **4** and C-5 for compounds **5** and **6**), while these of the quaternary carbons were 87.5, 86.8, 85.0 and 84.4 respectively (C-6 for compounds **3** and **4** and C-2 for compounds **5** and **6**) (Table 2).
These NMR data and MS spectra (Fig. 3) of compounds **3**-**6** and **8**-**15** allowed us to propose the following structures: **C** (C-3 and C-6 tetrahydrofuranoid) for compounds **3** and **4**; **D** (C-2 and C-5 tetrahydrofuranoid derivatives) for compounds **5** and **6**; **E** (C-2 and C-6 tetrahydropyranoid derivatives) for compounds **8-11** and **A** (tetraol alicyclic) for compounds **12**-**15** (Scheme 1). Structures **C**, **D** and **E** could have been generated by intramolecular attack and dehydration of tetraols (structure **A**) or through protonation followed by intramolecular attack of a possible intermediary (epoxide) (Scheme 1). This epoxide could be formed by oxidation of compound **1** and **2**.

Table 1. ^{1}H-NMR Spectroscopic Data (500 MHz) for compounds **4**-**17**

Proton	Furanoids				
	3	**4**	**5**	**6**	**7**
Me-1	1.16 s	1.16 s	1.28 s	1.32 s	1.22 s
H-3	3.86 dd (9.7, 3.2)[a]	3.94 ddd (9.3, 4.1, 0.8)	3.82 dd (5.8, 1.9)	3.74 dd (5.8, 1.1)	3.98 q (5.6)
H-4α	2.28 ddd (14.2, 9.5, 5.5)	2.42 ddd (14.2, 9.0, 5.5)	1.97 ddd (14.2, 4.5, 2.0)	1.89 ddd (14.5, 3.6, 1.1)	1.78 ddd (13.4, 7.3, 4.5)
H-4β	1.90 ddd (14.2, 3.2, 0.9)	2.00 ddt (14.2, 4.1, 1.3, 1.3)	2.44 ddd (14.4, 9.2, 5.8)	2.36 ddd (14.5, 9.5, 5.7)	2.47 ddd (13.4, 7.8, 6.3)
H-5	3.85 dd (5.5, 0.9)	3.90 dt (5.70, 1.3)	3.94 dd (9.0, 4.5)	3.91 dd (9.6, 3.6)	4.33 t (7.6)
H-7	5.81 dd (17.2, 10.7)	6.01 ddd (17.3, 11.0, 0.95)	6.04 dd (17.5, 10.9)	5.80 dd (17.3, 11.0)	5.78 ddquin (6.8, 6.7, 1.2)
H-8a	5.05 dd (10.7, 1.5)	5.25 dt (11.0, 1.4, 1.4)	5.17 dd (10.9, 1.1)	5.15 dd (11.0, 1.3)	4.20 d (6.3)
H-8b	5.25 dd (17.2, 1.6)	5.40 dt (17.5, 1.3, 1.3)	5.30 dd (17.4, 0.9)	5.33 dd (17.3, 1.3)	
Me-9	1.38 s	1.35 s	1.14 s	1.14 s	1.29 s
Me-10	1.32 s	1.23 d (0.95)	1.21 s	1.39 s	1.68 s

	Pyranoids			
	8	**9**	**10**	**11**
Me-1	1.28 s	1.32 s	1.26 s	1.20 s
H-3	3.55 dd (4.1, 3.8)	3.45 t (3.9)	3.89 d (9.5)	3.81 m
H-4α	2.05 ddd (14.3, 5.0, 4.9)	2.22 t (3.3)	1.96 dd (9.9, 3.0)	1.99 m
H-4β	2.23 dt (14.4, 3.2)	2.17 t (4.4, 4.4)	2.03 dd (5.7, 4.4)	
H-5	3.89 dd (4.1, 3.8)	3.64 dd (4.4, 2.8)	3.75 dt (5.3, 2.6)	4.06 m
H-7	6.04 dd (18.3, 11.7)	5.84 dd (17.3, 11.0)	5.83 dd (17.3, 10.7)	6.03 dd (18.0, 11.4)
H-8a	4.99 d (18.6)	5.33 dd (10.9, 1.7)	5.26 dd (11.0, 1.9)	5.04 d (18.4)
H-8b	5.02 d (11.7)	5.54 dd (17.3, 1.9)	5.47 dd (17.3, 1.9)	5.06 d (11.1)
Me-9	1.24 s	1.29 s	1.31 s	1.29 s
Me-10	1.30 s	1.35 s	1.36 s	1.24 s

	Tetraols					
	12:13		**14:15***		**16***	**17***
Me-1	1.18 s	1.17 s	1.15 s	1.14 s	1.15 s	1.13 s
H-3	3.64 m	3.62 m	3.56 dd (3.5, 2.2)	3.58 dd (3.4, 2.5)	3.59 dd (10.4, 1.9)	3.33 m
H-4a	a 1.44 m	a 1.47 m	1.55 m	1.55 m	a 1.47 ddd (14.2, 10.6, 2.7)	a 1.56 ddd (13.9, 10.5, 6.8)
H-4b	b 1.74 t (1.9 x 2)	b 1.71 t (1.9 x 2)			b 1.74 ddd (14.2, 9.8, 1.9)	b 1.83 ddd (13.9, 7.3, 1.9)
H-5	3.72 m	3.70 m	3.63 dd (5.4, 2.2)	3.65 dd (5.3, 2.2)	4.23 dd (9.8, 2.2)	4.24 t (7.1 x 2)
H-7	5.92 dd (17.3, 10.7)	5.93 dd (17.3, 11.0)	5.99 dd (17.5, 10.9)	5.98 dd (17.3, 11.0)	5.64 tquin (6.6, 1.3 x 2)	5.61 m
H-8a	5.21 dd (10.9, 1.4)	5.20 dd (10.9, 1.4)	5.12 dd (10.7, 1.6)	5.10 dd (10.7, 1.9)	4.14 d (6.6)	4.14 dd (6.5, 2.7)
H-8b	5.36 dd (17.3, 0.9)	5.33 dd (17.3, 1.3)	5.29 dd (17.3, 1.9)	5.28 dd (17.3, 1.6)		
Me-9	1.21 s	1.21 s	1.16 br. s	1.16 br. s	1.16 s	1.16 s
Me-10	1.27 s	1.32 s	1.26 s	1.25 s	1.67 d(0.9)	1.65 s

[a]overlapped with H-5 [3.85 dd (5.5, 0.9)]

*Methanol-d4

Table 2. ^{13}C-NMR Spectroscopic Data (500 MHz) for compounds **4**-**17**

Carbon	Furanoids				
	3	**4**	**5**	**6**	**7**
C-1	26.0	25.8	22.1	22.2	22.4
C-2	71.7	71.5	84.4	85.0	83.0
C-3	83.2	82.9	76.7	76.5	78.2
C-4	34.6	34.9	35.4	35.4	39.6
C-5	75.4	76.6	81.6	81.2	79.1
C-6	87.5	86.8	73.7	74.5	139.4
C-7	142.0	139.4	143.3	141.4	123.9
C-8	112.7	114.9	113.2	114.0	59.2
C-9	27.5	27.5	25.2	25.4	25.8
C-10	20.9	23.7	24.1	25.9	12.4
	Pyranoids				
	8		**9**	**10**	**11**
C-1	26.4		26.9	23.0	22.5
C-2	75.4		75.9	75.4	75.9
C-3	71.9		70.7	70.3	70.7
C-4	29.8		28.5	31.7	32.5
C-5	68.8		69.9	70.9	69.9
C-6	76.6		76.6	76.5	76.4
C-7	146.3		142.1	141.9	145.6
C-8	111.6		116.7	116.1	112.0
C-9	27.2		28.1	29.4	29.0
C-10	25.8		26.2	26.5	24.6
	Tetraols				
	12:13		**14:15***	**16***	**17***
C-1	23.8	23.7	25.6	25.5	25.3	24.9
C-2	72.7	72.8	73.8	73.8	73.8	73.6
C-3	78.7	78.9	76.0	75.9	76.0	78.1
C-4	31.6	31.0	34.3	34.3	38.2	37.0
C-5	77.8	78.2	75.5	75.3	74.6	77.6
C-6	75.3[a]	75.2	76.5	76.4	142.2	140.5
C-7	142.3	140.8	144.1	143.6	124.8	127.2
C-8	114.6	114.4	113.9	113.4	59.4	59.3
C-9	26.3	26.4	25.4	25.3	25.8	26.0
C-10	22.0	24.4	24.1	23.9	12.7	11.4

[a]no found in ^{13}C-NMR
*Methanol-d4

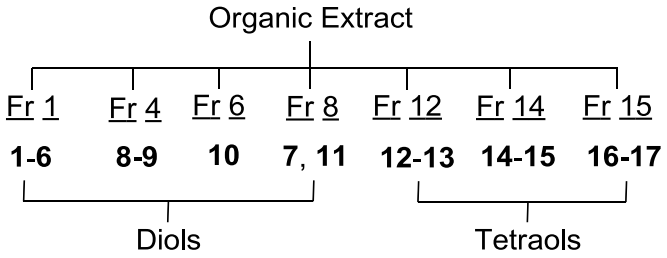


Figure 1. Fractionation and isolation scheme for *A. absinthium* organic extract (OE)

Figure 2. Fragments in common of compounds **3**-**17**. The numbering begins at the methyl as shown in **I** (in some cases the numbering is arbitrary)

The similarity between compounds **3** and **4** (Scheme 1, structure **C**) was confirmed by the fractionation pattern of their MS spectrum (Fig. 3), which was characterized by the presence of fragments C_3H_7O (hydroxyisopropyl group), C_3H_3O, C_4H_7O, C_5H_8O and $C_7H_{10}O$, suggesting that these compounds must be differenciated in the stereochemistry of the chiral centres.

Thus, the NOE correlation between the oxymethine proton H-3 (δ 3.86) with proton H-4β (δ 1.90) and the oxymethine proton H-5with H-4α (δ 2.28) of compound **3** indicated that H-3 and H-5 were on different sides of the molecule. Furthermore, the NOE correlation between Me-10 and H-4α suggested that Me-10 and H-5 are on the same side of the molecule. Assuming that the configuration at C-3 was not affected by intramolecular attack (Méou *et al.*, 1990), compound **3** should have a configuration (3S,5S,6R). The spectroscopic data of **3** was very similar to pantoisofuranoid A (Cueto *et al.*, 1998) and corresponded to a reported trans-4-hydroxylinalool 3,6-oxide, which absolute configuration at C-5 was not determined (Osorio *et al.*, 2002; Osorio and Duque, 2008). We have now confirmed the structure of **3** as (3S,5R,6R)-5-(2-hydroxypropan-2-yl)-2-methyl-2-vinyltetrahydrofuran-3-ol.

Unlike compound **3**, compound **4** showed NOE effects between H-3 (δ 3.94), H-5 (δ 3.90), Me-10 (δ 1.23)and H-4 (δ 2.42), suggesting that the hydroxyisopropyl, C-5 hydroxyl and vinyl groups are on the same side of the molecule. Assuming that the configuration at C-3 was not modified in the cyclation (Méou *et al.*, 1990), the molecule should have the configuration (3S,5S,6S). The NMR data did not agree with the stereoisomer *cis*-4-3,6-oxide hydroxylinalool (Osorio *et al.*, 2002; Osorio and Duque, 2008), but its mass spectrum was very similar. These data allowed us to identify **4** as (3S,5S,6S)-5-(2-hydroxypropan-2-yl)-2-methyl-2-vinyltetrahydrofuran-3-ol. Compound **4** has not been previously described.

Compounds **5** and **6** (Scheme 1, structure D) showed similar fragment pattern in their MS spectra (Fig. 3), characterized by the presence of fragments C_4H_7O and $C_6H_{11}O_2$. These compounds also had a furanoid structure, but unlike **3** and **4**, the oxygen was bound between carbons C-2 and C-5. The relative configuration of these compounds was determined by 2D-NOESY experiments.

Compound **5** showed NOE between H-3 (δ 3.82), H-5 (δ 3.94) and H-4 (δ 2.44) and these protons with the methyl (δ 1.14) at C-2, indicating that H-3 and H-5 were on the same side of the molecule and the fragment C_4H_7O (2-hydroxybut-3-en-2-yl)was in *cis* position relative to the hydroxyl group at C-3 (δ 76.5).The protons Me-10(δ 1.21) showed a strong interaction with protons Me-1 (δ 1.28) and Me-9 (δ 1.14) suggesting that the Me-10 is oriented towards the oxygen of the furanoid ring and between the geminal methyl groups. The lack of interactions between the vinyl group protons and H-4α or Me-1, and the small NOE effects between H-7 (δ 6.04) and H-5 suggested that the hydroxyl group at C-6 (δ 73.7) was closer to the ring than the vinyl group. Assuming that the C-3 configuration was affected at the cyclation process, compound **5** should have an R configuration at C-6. Therefore, **5** was identified as (3S, 5S, 6R)-5-(2-hydroxybut-3-en-2-yl)-2,2-dimethyltetrahydrofuran-3-ol.

The NOESY experiments of **6** also showed that the fragment C_4H_7O (2-hydroxybut-3-en-2-yl) and the hydroxyl group were in *cis* position.Therefore, the difference between **5** and **6** is due to the carbon C-6 (δ 74.5) configuration. The data reported for a similar halogenated compound (Cueto and Darias, 1996), and the assumption of a configuration S to C-3 allowed us to identify **6** as (3S,5S,6S)-5-(2-hydroxybut-3-en-2-yl)-2,2-dimethyltetrahydrofuran-3-ol.

Compounds **8** to **11** (Scheme 1, structure E) showed a similar fragmentation pattern (Fig. 3) characterized by fragments C_2H_3O, $C_3H_4O_2$ and C_5H_8O. The relative configurations of these compounds were determined by 2D-NOESY experiments.

The NOESY spectrum of **8** showed correlations between H-4α (δ 2.05), Me-1 (δ 1.28) and Me-10 (δ 1.30); between Me-9 (δ 1.24), H-3 (δ 3.55), H-4β (δ 2.23) and H-7 (δ 6.04) and between Me-10 (δ 1.30) and H-5 (δ 3.89). These data suggested that the vinyl and the hydroxyl group at C-5 (δ 68.8) were in *trans* position respect to the hydroxyl group at C-3 (δ 71.9). Therefore, compound **8** was identified as (3S,5S,6S)-2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3,5-diol.

The NOESY spectrum of **9** showed correlations between H-4α (δ 2.22) and Me-9 (δ 1.29) and Me-10 (δ 1.35), between H-5 (δ 3.64), 4H-β (δ 2.17) and Me-1 (δ 1.32) and H-3 (δ 3.45) with Me-9 (δ 1.29). These data suggested that the vinyl and the hydroxyl group at C-5 (δ 69.9) were in *trans* position respect to the hydroxyl group at C-3 (δ 70.7). Therefore, compound **9** was identified as (3S,5S,6R)-2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3,5-diol.

The NOESY spectrum of **10** showed correlations between H4α (δ 1.96), Me-1 (δ 1.26) and Me-10 (δ 1.36) and between Me-9 (δ 1.31), H-3 (δ 3.89), H-4β (δ 2.03) and H-7 (δ 5.83). Additional correlations between the oxymethine protons H-3 (δ 3.89) and H-5 (δ 3.75) were observed. These data suggested that bothhydroxyl groups were on the same side of the ring with the vinyl group in *trans* position. Therefore, **10** was identified as (3S,5R,6S)-2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3,5-diol.

The NOESY spectrum of **11** showed correlations between Me-1 (δ 1.20) and H-7 (δ 6.03); the oxymethine proton H-3 (δ 3.81) with Me-9 (δ 1.29); between Me-10 (δ 1.24) and the oxymethine proton H-5 (δ 4.06) and the correlation between the oxymethine protons H-3 (δ 3.81) and H-5 (δ 4.06). These data suggested that both hydroxyl groups were on the same side of the ring with the vinyl group in *cis* position. Therefore, **11** was identified as (3S,5R,6R)-2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3,5-diol.

From the most polar fraccions, were obtained compounds **12**-**15** as 1:1 mixtures of diastereomeric oxygenated monoterpenes **(12**:**13** and **14**:**15**). The EIMS of these compounds showed similar fractionation patterns characterized by the presence of fragments C_4H_7O, C_5H_8O, C_3H_7O (hydroxyisopropyl) and $C_6H_{11}O_2$. Its molecular formula $C_{10}H_{20}O_4$ indicated a degree of unsaturation corresponding to the double bond between C-7 and C-8 observed in the ^{1}H- and ^{13}C-NMR spectra.The analysis ^{1}H-NMR, ^{13}C-NMR, HSQC and HMBC experiments allowed us to assign the hydrogen signals with their corresponding carbon signals for each mixture (Tables 2 and 3) and to indentify these new compounds as stereoisomers of 2,6-dimethyloct-7-ene-2,3,5,6-tetraol. The main difference between these diastereomer mixtures was the higher polarity of **14**:**15**.

The NMR data of compounds **16** and **17** showed similar ^{1}H-NMR and ^{13}C-NMR spectra (Tables 1 and 2) to those of compund **7**, suggesting that these compounds were related. The molecular formula of these compounds was determined by HRESI-TOFMS as $C_{10}H_{18}O_3Na$, indicating the presence a degree of unsaturation in their structures. The major difference between these compounds and the oxygenated monoterpenes described above was the absence of the terminal double bond (vinyl group) and the presence of a trisubstituted double bond (C-6/C-7: *E*-configuration).
The ^{1}H-NMR spectra showed signals of three methyls, two of them on a quaternary oxygenated carbon (Me-1 and Me-9) and the other on a double bond (Me-10); two aliphatic methylenes (H-4a, H-4b and H-8) the latter belonging to a primary alcohol; three methines, two oxymethines (H-3 and H-5) and one olefinic (H-7). Their ^{13}C-NMR spectra confirmed the presence of three methyls (C-1, C-9 and C-10), two methylenes (C-4 and C-8), three methines (C-3, C-5 and C-7) and signals corresponding to two oxygenated quaternary carbons (C-2 and C-6). The ^{1}H-^{1}H COSY spectrum showed correlations between aliphatic methylene protons (H-4a and H-4b) and oxymethine protons (H-3 and H-5) and between the olefinic methylene proton (C-7) and oxygenated methylene protons (C-8). The 2D NMR experiments (HSQC and HMBC) of compounds **7**, **16** and **17** showed significant correlations between Me-1 protons and carbons C-2, C-3 and C-9; the oxymethine proton H-5 with carbons C-6, C-7 and C-10; between the olefinic proton H-7 with carbons C-5, C-6 and C-10 and of Me-10 protons and carbons C-5, C-6 and C-7. Considering the information described above, compounds **7**, **16** and **17** have connectivity shown fragment II (Fig 1). Therefore, these compounds had a linear structure and were identified as two of the possible stereoisomers of (*E*)-3,7-dimethyloct-2-ene-1,4,6,7-tetraol. The structure of sachalinol C (**7**) (Fan, 2001; Simon, 2011) could have been formed by an intramolecular attack and dehydration of tetraols **16** or **17** (Scheme 1).

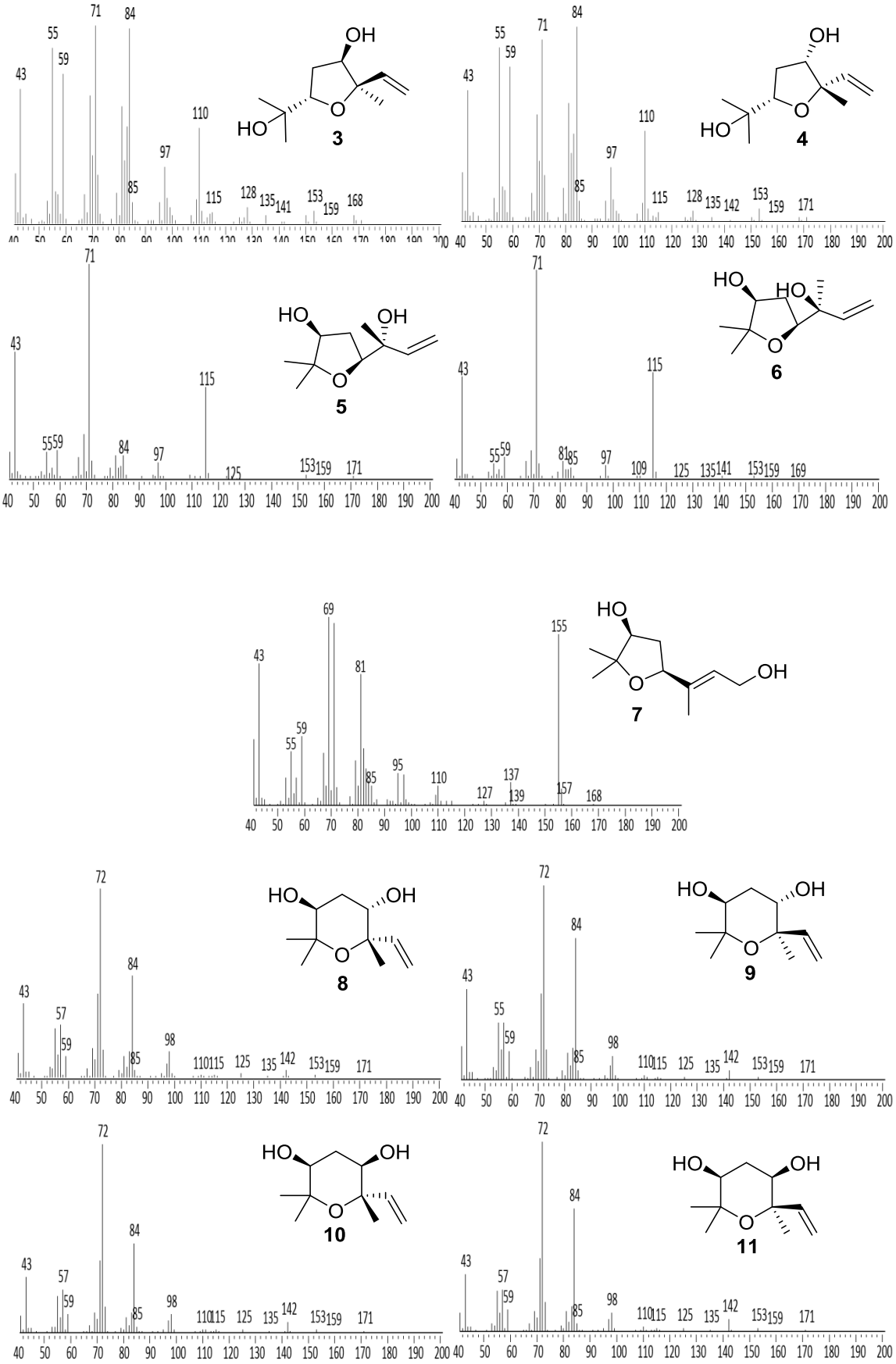
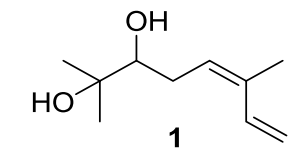
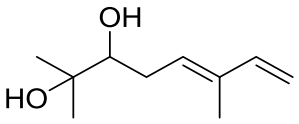


Figura 3. Mass spectra (GC/MS, EI 70 eV) and chemical structure of compounds **4**-**11.**

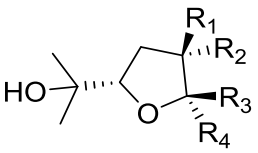
The essential oil and the aqueous byproduct from the solid phase extraction of the hydrolate (WR) were not nematicidal, while the organic extract (OE) showed a strong effect (Table 3). Among the compounds tested, *cis*-2,6-dimethylocta-5,7-dien-2,3-diol (**1**) was the most active followed by the one of the isomers of (E)-3,7-dimethyloct-2-ene-1,4,6,7-tetraol (**16**). The activity of the organic extract is probably due to the main component, **1**, showing the highest nematicidal effects.

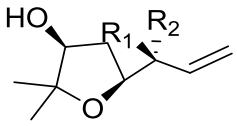


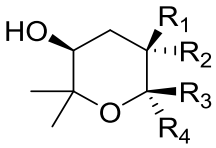
1



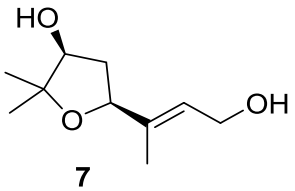
2



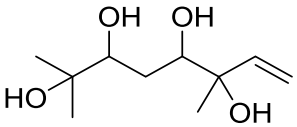




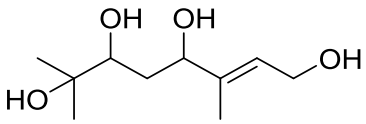
	R_1	R_2	R_3	R_4
3	OH	H	$CH=CH_2$	CH_3
4	H	OH	CH_3	$CH=CH_2$
5	CH_3	OH		
6	OH	CH_3		
8	H	OH	CH_3	$CH=CH_2$
9	H	OH	$CH=CH_2$	CH_3
10	OH	H	CH_3	$CH=CH_2$
11	OH	H	$CH=CH_2$	CH_3



7



12:**13** and **14**:**15**



16 and **17**

Compound **1**, isolated from *A. absinthium*, had moderate antifeedant effect on the insect *Spodoptera littoralis*(Bailen *et al.*, 2013). The nematicidal activity (against *Caenorhabditis elegans*) of a series of monoterpenoids including some acyclic alcohols such as nerolidol, geraniol, citronellol and farnesol, has been reported (Abdel-Rahman *et al.*, 2012). However, this is the first report on the nematicidal activity of **1** and the tetraol **16**. The fact that compounds **2** or **17** (isomers of **1** and **16**) were not active indicates a structure-dependent effect of these type of compounds. Given the abundance of **1** in the residual hydrolate and its nematicidal effect, this compound can be considered the chemical biomarker of this extract and its potential formulations. Therefore, the hydrolates from essential oil production are a potential source of hydroxylated nematicidal compounds (Julio *et al.*, under revision).

Tabla 3. Nematicidal activity of *Artemisia absintium* (var candial) hydrolate, (100%), the organic fraction (1mg/mL) and their compounds (0.5 mg/mL) against infective juveniles (J2) of *Meloidogyne javanica*.

	% J2 Mortality[b]	DL_{50}	DL_{90}
WRT08	100.0 ± 0.0[a]	0.30 (0.30 - 0.31)	0.36 (0.35 - 0.38)
OE	91.4 ± 6.5[a]		
1	100 ± 0.0	0.24 (0.22-0.26)	0.40 (0.38-0.44)
3	8.4 ± 1.6		
4	4.0 ± 0.6		
6	5.6 ± 1.5		
7	2.0 ± 0.9		
8	1.8 ± 0.9		
9	2.7 ± 1.2		
10	0.2 ± 0.7		
11	4.2 ± 1.0		
12:13	2.2 ± 0.7		
14:15	2.1 ± 0.5		
16	75.5 ± 2.9		
17	5.2 ± 1.0		

[a] Data published in Julio et al 2015

[b]Mortality was observed 72 h after treatment. Values are means of four replicates and corrected according to Scheider–Orelli's formula.

[c]Five concentrations were used to obtain DL_{50} and DL_{90}. CL denotes confidence limit

The hydrolate was phytotoxic to *Lactuca sativa* and *Lolium perenne* but the organic fraction (OE) was not (Julio et al., in preparation a). Among the compounds tested (**1, 3, 4, 6-8, 10, 16, 17, 12:13, 14:15**) some showed moderate-low phytotoxic effects against *L*. *sativa* and *L*. *perenne*. Compounds **6**, **10** and **16** reduced *L*. *sativa* root growth (11-29%). Compounds **6**-**8**, **10**, **17** andmixtures **12**:**13** and **14**:**15** inhibited the germination of *L*. *perenne* (25-50%) during the first 72 hours and showed moderate growth inhibition (21-28%) of the cotyledon.

Conclusions

Here we present the chemical characterization of a nematicidal byproduct (hydrolate) obtained from the extraction of essential oil by vapor-pressure steam distillation of a domesticated population of Spanish *Artemisia absinthium* (var. Candial). The study of the compounds present in the bioactive organic fraction of the hydrolate allowed for the identification of seventeen monoterpene compounds: two known monoterpene diols: (Z) and (E)-2,6-dimethylocta-5,7-diene-2,3-diol (**1** and **2**), five tetrahydrofuranoid isomer derivatives (**3**-**7**), four tetrahydropyranoid isomer derivatives (**8**-**11**), and six tetraols (**12**-**17**). Compounds **4**-**6** and **8**-**17** have not been previously described. Among the tetrahydrofuranoids, compounds **3** and **4** were stereoisomers of 5-(2-hydroxypropan-2-yl)-2-methyl-2-vinyltetrahydrofuran-3-ol. Compounds **5** and **6** were stereoisomers of 5-(2-hydroxybut-3-en-2-yl)-2,2-dimethyltetrahydrofuran-3-ol. The four tetrahydropyranoid derivatives (**8**-**11**) were stereoisomers of 2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3,5-diol. Among the more polar

tetraols (**12**-**17**), four of them were stereoisomers of 2,6-dimethyloct-7-ene-2,3,5,6-tetraol (mixtures 1:1 of 12:13 and 14:15) and two isomers of (E)-3,7-dimethyloct-2-ene-1,4,6,7-tetraol (16 and 17). Compounds **4**-**6** and **8**-**17** have not been previously described. Compound **1** showed nematicidal effects against *Meloidogyne javanica.*

Material and Methods

General experimental procedures

Optical rotations were determined in $CHCl_3$ at room temperature using a Perkin-Elmer 343 polarimeter. NMR spectra were measured on a Bruker AMX2 500 MHz spectrometer with pulsed field gradient using chloroform and methanol as internal standard (CDCl3, at δH7.26 and δC77.0; MeOD, at δH3.31 and δC49.1). The programs used in two-dimensional (2D) NMR experiments (HMBC, HSQC, COSY, and NOESY) were those furnished with the manufacturer's software. HRESI MS (positive-ion mode) data were obtained using a Micromass LCT Premier and EIMS were recorded on a Micromass Autospec instrument at 70 eV. GC-MS were determined using an Agilent 6890N GC (Agilent Technologies, USA) coupled to an Agilent 5973N mass detector (electron ionisation, 70 eV) (Agilent Technologies) and equipped with a 25 m × 0.20 mm i.d. capillary column (0.2 µm film thickness) HP-1 (methyl silicone bonded) (Hewlett-Packard). Activated Carbon (granulate, Scharlau) was used as adsorbent. Preparative flash chromatography was carried out on a column 5 cm in diameter with a height of 22 cm and 2.5 cm diameter silica cartridges (40-70 µm) in a Jones Flash Chromatography apparatus. Semipreparative HPLC was carried out on a Shimadzu LC-20AD HPLC (ACE 5 SIL 250 mm x 10 mm, 5 um particle size). Silica gel 40-70 µm (Merck) was used for column chromatography. Compounds were visualized on TLC with Oleum reagent.

Plant material and cultivation

The individuals for field cultivation were obtained from seeds of two populations (Teruel, T1 and Sierra Nevada, SN0) and planted in 2008 to give the domesticated populations T2 and SN1 (Julio et al. 2015). The experimental field is located in Ejea de los Caballeros, Zaragoza, Spain. A detailed description of the field and the cultivation parameters has been reported (Burillo, 2009).

Extraction and fractionation

Semi-industrial vapor pressure extraction (VP extracts) was carried out in a stainless steel industrial distillation plant (http://www.ecoaromuz.com) equipped with two 3,000 L vessels. The hydrolate (WR) was obtained as an EO-free aqueous residue in the process of extraction of essential oils. 5 L of WR were extracted by solid phase extraction using 500 g of activated carbon (activated charcoal granulate, Scharlau) as adsorbent material. Activated carbon and WR was stirred for 30 minutes. The aqueous portion was discarded and the activated carbon was fully dried in an oven with air flow at 40 °C for 24 h. The adsorbed organic material was extracted by continuous solvent extraction (Soxhlet, ethanol) for 4 hours. The eluent was filtered and dried under reduced pressure to give 5.1 g (1.0 g / L) of an oily extract.

5 g of the organic extract were separated by normal phase chromatography using silica gel (160 g Si, 35-70 µm), a column 5 cm in diameter with a height of 22 cm, increasing polarity CH_2Cl_2-MeOH (0 -15%, 50 min, 15%, 10 min; 15-25%; 5 min; 25%, 10 min) and a flow of 70 mL / min. The fractions obtained (15) were separated by flash chromatography using a 20 g Si pre-packed flash cartridge (ExtraBond Flash OT SI 20g 70 mL 26.8 x 154 mm Scharlau) at a flow of 18 mL / min., and by semipreparative HPLC using a Shimadzu LC-20AD HPLC (ACE 5 SIL 250 mm x 10 mm, 5 µm particle size) and mixtures of CH_2Cl_2-MeOH or CH_2Cl_2-EtOAc at a flow rate of 4 mL / min to give compounds **1**-**17**.
Compound **1**was also obtained by acid hydrolysis ofa *cis*-epoxyocimene enriched *A. absinthium* essential oil fraction (Julio *et al.*, 2015). 500 mg of the oil fraction were added to 15 mL of acidified distilled water (HCl, pH 2.), the mixture was stirred for 30 minutes, filtered and extracted (CH_2Cl_2, 15 mL x 2). The organic extract was dried over anhydrous Na_2SO_4 and concentrated to dryness to give 222 mg of organic extract. This extract was fractionated by column chromatography (Flash) using a 20 g prepacked cartridge Si (ExtraBond Flash OT SI 20g 70 mL 26.8 x 154 mm Scharlau) at a flow of 18 mL / min. and a mixture of CH_2Cl_2-MeOH (3%, isocratic) to give 153 mg of compound **1** (yield; 31.8%).

Compound 3. $[\alpha]_D$: +45.2 $CHCl_3$ (c 0.25); HRESI-TOFMS m/z 209.1159 $[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 110 (48), 84 (99), 83 (49), 81 (59), 71 (100), 70 (35), 69 (65), 59 (76), 55 (90), 43 (69); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3

Compound 4. $[\alpha]_D$: +11.5 $CHCl_3$ (c 0.15); HRESI-TOFMS m/z 209.1161 $[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 110 (46), 84 (100), 83 (45), 82 (35), 81 (61), 71 (93), 69 (55), 59 (80), 55 (90), 43 (68); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 5. HRESI-TOFMS m/z 209.1152 $[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 115 (41), 81 (11), 72 (9), 71 (100), 69 (18), 67 (9), 59 (14), 55 (12), 43 (81), 41 (19); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 6. $[\alpha]_D$: +25.6 $CHCl_3$ (c 0.39); HRESI-TOFMS m/z 209.1161 $[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 115 (51), 81 (8), 72 (8), 71 (100), 69 (13), 67 (8), 59 (10), 55 (8), 43 (50), 41 (9); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 7. HRESI-TOFMS m/z 209.1149$[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 155 (90), 82 (30), 81 (70), 71 (97), 69 (100), 67 (28), 59 (37), 55 (30), 43 (78), 41 (37). ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 8. $[\alpha]_D$: -2,5 $CHCl_3$ (c 0,22); HRESI-TOFMS m/z 209.1156 $[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 98 (14), 84 (54), 83 (14), 73 (15), 72 (100), 71 (45), 69 (16), 57 (28), 55 (27), 43 (40);^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 9. [α]D : -6.8 $CHCl_3$ (c 0.2); HREIMS m/z 169.1228 $[M-OH]^+$, (calcd for $C_{10}H_{17}O_2$, 169.1229); EIMS 70 eV m/z (rel. int.): 84 (78), 83 (16), 73 (15), 72 (100), 71 (43), 57 (29), 56 (15), 55 (30), 43 (45), 41 (16); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 10. HREIMHRESI-TOFMS m/z 209.1152 $[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 84 (48), 83 (11), 73 (14), 72 (100), 71 (39), 69 (11), 59 (11), 57 (23), 55 (20), 43 (30); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 11. HRESI-TOFMSm/z 209.1153 $[M+Na]^+$, (calcd for $C_{10}H_{18}O_3Na$, 209.1154); EIMS 70 eV m/z (rel. int.): 84 (65), 83 (13), 73 (16), 72 (100), 71 (38), 69 (11), 59 (11), 57 (22), 55 (22), 43 (30); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 12. HRESI-TOFMSm/z 227.1262 $[M+Na]^+$, (calcd for $C_{10}H_{20}O_4Na$, 227.1259); EIMS 70 eV m/z (rel. int.): 169 (46), 151 (20), 115 (28), 113 (19), 84 (64), 83 (30), 72 (46), 71 (100), 59 (43), 55 (31); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 13. HRESI-TOFMSm/z 227.1262 $[M+Na]^+$, (calcd for $C_{10}H_{20}O_4Na$, 227.1259); EIMS 70 eV m/z (rel. int.): 169 (46), 151 (20), 115 (28), 113 (19), 84 (64), 83 (30), 72 (46), 71 (100) 59 (43), 55 (31); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 2; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3.

Compound 14. HRESI-TOFMS m/z 227.1264, (calcd for $C_{10}H_{20}O_4Na$, 227.1259); EIMS 70 eV m/z (rel. int.): 169 (46), 151 (20), 115 (28),113 (19), 84 (64), 83 (30), 72 (46), 71 (100), 59 (43), 55 (31); ^{1}H NMR data (MeOD, 500 MHz) see Table 2; ^{13}C NMR data (MeOD, 125 MHz) see Table 3.

Compound 15. HRESI-TOFMS m/z 227.1264, (calcd for $C_{10}H_{20}O_4Na$, 227.1259); EIMS 70 eV m/z (rel. int.): 169 (46), 151 (20), 115 (28), 113 (19), 84 (64), 83 (30), 72 (46), 71 (100), 59 (43), 55 (31); ^{1}H NMR data (MeOD, 500 MHz) see Table 2; ^{13}C NMR data (MeOD, 125 MHz) see Table 3.

Compound 16. [α]D: -11.9 EtOH (c 0.86); HRESI-TOFMS m/z 227.1257, (calcd for $C_{10}H_{20}O_4Na$, 227.1259); EIMS 70 eV m/z (rel. int.): 168 (4), 155 (15), 110 (17), 109 (8), 97 (28), 95 (15), 84 (66), 81 (75), 71 (85), 59 (100),55 (51); ^{1}H NMR data (MeOD, 500 MHz) see Table 2; ^{13}C NMR data (MeOD, 125 MHz) see Table 3.

Compound 17. $[\alpha]_{D:}$ 0.7 $CHCl_3$ (c 0.96); HRESI-TOFMS m/z 227.1262, (calcd for $C_{10}H_{20}O_4Na$, 227.1259); EIMS 70 eV m/z (rel. int.): 168 (3), 155 (12), 110 (14), 109 (5), 97 (26), 95 (14), 84 (62), 81 (68), 71 (72), 59 (100), 55(30); ^{1}H NMR data (MeOD, 500 MHz) see Table 2; ^{13}C NMR data (MeOD, 125 MHz) see Table 3.

Nematode bioassays

A *Meloydogine javanica* population maintained on *Lycopersicon esculentum* plants (var. Marmande) in pot cultures at 25 ± 1 °C, > 70% relative humidity has been used. Second stage juveniles (J2) hatched within a 24 h period from egg masses handpicked from infected tomato roots were used.The experiments were carried out in 96-well microplates (Becton, Dickinson) as described (Andres *et al.*, 2012). Number of J2 dead was recorded after 72 hours. All treatments were replicated four times. The data is presented as percent mortality corrected according to Scheider-Orelli's formula.

Phytotoxic activity

The experiments were conducted with *Lactuca sativacv* Teresa(Fito, España) and *L. perenne* seeds as described. (Martín *et al.*, 2011) Germination was monitored for 6 days and the rootlet length measured at the end of the experiment (25 plantlets randomly selected for each experiment, digitalized and measured with the application Image J, http//rsb.info.nih.gov./ij/). A non-parametric analysis of variance (ANOVA) was performed on radical length data.

ACKNOWLEDGEMENTS
This work has been supported by grant CTQ2012-38219-C03-01 (Spain), and JAE-CSIC (predoctoral fellowship to L.F. Julio).

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4.5. Trypanocidal, trichomonacidal and cytotoxic components of cultivated *Artemisia absinthium* Linnaeus (Asteraceae) essential oil

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Mem Inst Oswaldo Cruz, Rio de Janeiro (2015), 110, 693-699.
doi: 10.1590/0074-02760140129

Artemisia absinthium is an aromatic and medicinal plant of ethnopharmacological interest and it has been widely studied. The use of A. absinthium based on the collection of wild populations can result in variable compositions of the extracts and essential oils (EOs). The aim of this paper is the identification of the active components of the vapour pressure (VP) EO from a selected and cultivated A. absinthium Spanish population (T2-11) against two parasitic protozoa with different metabolic pathways: Trypanosoma cruzi and Trichomonas vaginalis. VP showed activity on both parasites at the highest concentrations. The chromatographic fractionation of the VP T2-11 resulted in nine fractions (VLC1-9). The chemical composition of the fractions and the antiparasitic effects of fractions and their main compounds suggest that the activity of the VP is related with the presence of trans-caryophyllene and dihydrochamazulene (main components of fractions VLC1 and VLC2 respectively). Additionally, the cytotoxicity of VP and fractions has been tested on several tumour and no tumour human cell lines. Fractions VLC1 and VLC2 were not cytotoxic against the nontumoural cell line HS5, suggesting selective antiparasitic activity for these two fractions. The VP and fractions inhibited the growth of human tumour cell lines in a dose-dependent manner.

Key words: Artemisia absinthium - essential oil - activity - Trypanosoma cruzi - Trichomonas vaginalis – cytotoxicity.

INTRODUCTION

The Artemisia genus belongs to the family Compositae (Asteraceae) and consists of about 500 species distributed through the world. Artemisia absinthium L. is an aromatic and medicinal plant of ethnopharmacological interest (Bora & Sharma 2010, Lachenmeier 2010).The composition and biological effects of the essential oil (EO) and the extracts of A. absinthium have been widely studied. Different researches have demonstrated its antimicrobial and antiprotozoal effects against Leishmania aethiopica, Leishmania donovani, Leishmania infantum and Trypanosoma cruzi (Juteau et al. 2003, Tariku et al. 2011, Erel et al. 2012, Nasrabadi et al. 2012, Bailén et al. 2013, Bachrouch et al. 2015). Among the major A. absinthium EO components reported are α and β-thujone, myrcene, trans-sabinyl acetate, β-pinene, 1,8-cineole, camphor, cis-epoxyocimene, chrysanthenyl acetate, sabinene, myrtenol, bornyl acetate, artemisiaketone, linalool, hydrocarbon monoterpenes, sesquiterpene lactones (Pino et al. 1997, Jaenson et al. 2005, Kordali et al. 2005, Geszprych et al. 2010, Martín et al. 2011, Erel et al. 2012, Judzentiene et al. 2012, Sharopov et al. 2012, Tehrani et al. 2012, Umpiérrez et al. 2012) and mixtures of some of these components, depending on the plant origin (Chialva et al. 1983, Carnat et al. 1992, Geszprych et al. 2010, Bailén et al. 2013). In fact, the use of A. absinthium based on the collection of wild populations can result in variable compositions of the extracts and EOs. A. absinthium is abundant in the mountains of the Iberian Peninsula, where seven chemotypes have been described (Ariño et al. 1999). Two Spanish populations of wormwood have been domesticated for experimental cultivation in the field and under controlled conditions (Burillo 2009, Martín et al. 2011, González-Coloma et al. 2012a). Based on these results, a long-term field cultivation of selected A. absinthium plants has been established for further valorisation of its extracts. The aim of the present paper is the identification of the active components of the vapour pressure (VP) EO from a selected and cultivated A. absinthium Spanish population against two parasitic protozoa with different metabolic pathways, T. cruzi and Trichomonas vaginalis. T. cruzi is the aetiologic agent of Chagas disease, a frequently fatal illness affecting the heart and gastrointestinal systems. An estimated eight million people in Latin America are infected with this pathogen and it is also spreading to the United States of America, Canada and many parts of Europe and the Western Pacific as a result of migratory flows (Rassi Jr et al. 2010). Only two drugs, nifurtimox (NFX) and benznidazole are in use against chronic infections and both have limitations, due to the need of a large number of doses over a long time period, side effects and lack of effectiveness against all stages of the disease and all strains of the parasite. Moreover, their lack of efficiency has involved problems in their production and distribution (González-Coloma et al. 2012b). T. vaginalis is a parasitic protozoa and a major cause of vaginitis, cervicitis and urethritis in women and may cause nongonococcal urethritis, prostatitis and other genitourinary tract syndromes in men. Trichomoniasis is among the world's most common sexually transmitted diseases with an annual incidence of more than 276 million cases per year (WHO 2012). Its clinical manifestations vary from asymptomatic infection to an acute vaginitis. A single drug, metronidazole, is currently available for treating trichomoniasis. However, metronidazole resistant strains have been found in unsuccessfully treated patients and some adverse effects have been described (Dunne et al. 2003, Cudmore et al. 2004). Natural products could be a source of new drugs. In this paper the antiparasitic effects of a characterised A. absinthium VP EO against T. cruzi and T. vaginalis will be discussed along with the chemical composition of the active fractions. Additionally, their selective cytotoxicity has been tested on several tumoural (A549, H292, HCT116, MCF7, SK-MEL5) and nontumoural (HS5) human cell lines.

MATERIALS AND METHODS

Plant material and cultivation - The individuals for field cultivation were obtained from selected seeds (A. absinthium var. candial®) and planted in Ejea de los Caballeros (Zaragoza, Spain) in 2008. A detailed description of the field and the cultivation parameters has been reported (Burillo 2009). Flowering plants were harvested yearly and processed for VP extraction. The material under study is endotoxin free. EO analysis - Plant material was distilled in an industrial stainless steel VP extraction plant equipped with two 3000 L vessels (ecoaromuz.com). The VP EO extracted was analysed by gas chromatography mass spectrography (GC-MS) using an Agilent 6890N GC (Agilent Technologies, USA) coupled to an Agilent 5973N mass detector (electron ionisation, 70 eV) (Agilent Technologies) and equipped with a 25 m × 0.20 mm i.d. capillary column (0.2 µm film thickness) HP-1 (methyl silicone bonded) (Hewlett-Packard). Working conditions were as follows: split ratio (20:1), injector temperature 260ºC, temperature of the transfer line connected to the mass spectrometer 280ºC, initial column temperature 70ºC, then heated to 270ºC at 4ºC min-1. Electron ionisation mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001). Quantitative data were obtained from the total ion current peak areas without the use of response factors. Fractionation of the EO - A VP extract of A. absinthium (20 g, T2 population, 2011) was submitted to vacuum liquid chromatography on a Si-gel column (40-70 µm, 6 cm diameter, 9 cm length) eluted with a hexane (Hx):dichloromethane (DCM) gradient of increasing polarity (0.5-100% DCM).

Nine fractions were obtained and analysed by GC-MS as described. Fraction 8 (690 mg) was further purified by flash chromatography on a 2.5 cm diameter silica cartridge (40-70 µm) eluted with a Hx:DCM (70:30) mixture (isocratic, 18 mL/min flow rate) and by Sephadex LH-20 chromatography [DCM: methanol (MeOH), 1:1] to give (-)-cischrysanthenol (4) (33.5 mg; 4.9%). All solvents used were of analytical grade. n-Hx, DCM and MeOH were obtained from Lab-Scan Analytical Sciences (Poland). Silica gel (70-30 mesh) and thin layer chromatography plate (silica gel 60F254) was purchased from Merck Co (Germany). (-)-cis-chrysanthenol was isolated from A. absinthium EO (VP extract) and trans-caryophyllene 80% was purchased from Sigma-Aldrich (USA). The VP EO is constituted by apolar substances and the activity assays are performed in aqueous culture media. To the tests, VP, fractions and products are previously dissolved in acetone at an initial concentration of 10 mg/mL. From this initial solution, the different concentrations for assays are prepared using the culture medium of cells and parasites. Trypanocidal in vitro activity - Trypanocidal activity was assayed on epimastigote forms of T. cruzi Y strain, cultured in liver infusion tryptose medium supplemented with 10% heat-inactivated foetal calf serum. Parasites in logarithmic growth phase from an initial culture with 2 × 106 epimastigotes/mL were distributed in 96-well flat-bottom plates. Each well was filled with 90 µL of culture after two days of incubation. VP, factions and compounds were tested at several concentrations (VP at 800, 400, 200, 100, 10 and 1 µg/mL; fractions and compounds at 100, 10 and 1 µg/mL) for 72 h. NFX was used as the reference drug and parasite viability was analysed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay method (González-Coloma et al. 2012b). Briefly, after 72 h, 10 µL MTT/phenazine methosulfate were added to each well. Incubate 75 min to occur the reduction of MTT and 100 µL sodium dodecyl sulphate were added to dissolve formazan crystals obtained as a result of the reduction of MTT. Once the crystals have dissolved (15-30 min), the plate was read on a spectrophotometer at 570 nm. The activity was calculated as percentage growth inhibition (%GI) as follows: %GI = 100 - [(Ap - Ab)/(Ac - Ab)] x 100, where Ap being the absorbance of problem wells (treated), Ac the absorbance

of control wells (not treated) and Ab the absorbance of blank wells (culture medium and vehicle only). All assays were carried out in triplicate and were repeated at least three times independently to confirm the results. The concentration that inhibits 50% the growth of the parasites (GI50) as well as the 95% confidence intervals (CIs) were calculated by Probit analysis (SPSS v.20, IBM). Activity on T. vaginalis - Trichomonacidal assays were carried out against the metronidazole-sensitive T. vaginalis JH31A no.4 isolate [American Type Culture Collection, (ATCC)]. The flagellates were cultured in trypticase-yeast extract-maltose modified medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) and antibiotic solutions at 37ºC and 5% CO2 . Assays were carried out in glass tubes containing 105 trophozoites/mL. After 5-6 h of seeding, the VP, factions and compounds were added to log-phase growth cultures at several concentrations (500, 250, 100, 75, 37.5 and 18.75 µg/mL). The tubes were incubated for 24 h at 37ºC and 5% CO2 . The trichomonacidal activity was obtained by a fluorimetric method using resazurin (SigmaAldrich) as previously described (Escribano et al. 2012). The experiments were performed at least two times in triplicate. GI50 values as well as the 95% CI were calculated by Probit analysis (SPSS v.20, IBM).

Cytotoxicity assays - The A549, NCI-H292 (adenocarcinoma and squamous non-small cell lung cancer, respectively), HCT116 (colorectal carcinoma), MCF7 (luminal breast adenocarcinoma), SK-MEL-5 (melanoma) and HS5 (bone marrow stromal) human cell lines were employed to determine the toxicity of the VP and fractions. HS5 was used as nontumour control cells. Cell lines were purchased from LGC Promochem, SLU-ATCC (Spain). All cell lines were propagated in RPMI-1640 medium, supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine (Lonza Verviers). Cells were grown at 37ºC in a humidified atmosphere with 5% CO2 and were in the logarithmic growth phase at the initiation of the experiments. For the determination of the activity, cells diluted in 100 µL/well of complete cell culture medium were plated in 96-well flat-bottom plates and allowed to attach for 24 h. Growth medium was removed from the wells and replaced by medium containing the VP or fractions at concentrations of 100 and 250 µg/mL for another 72 h. The anticancer drugs paclitaxel (a cyclic diterpene) and cisplatin (drug based on the platinum) were tested as reference products. All experimental points were set up in four wells and all were confirmed in at least three independent experiments. Viable cells were determined using the WST-1 assay (Roche, Germany) according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Table I shows the composition of the VP and its fractions. cis-epoxyocimene was the major component (40%) followed by (-)-cis-chrysanthenol (12%), dihydrochamazulene (6%) and chrysanthenyl acetate (5.3%). Camphor (4.5%), trans-caryophyllene (4%) and chamazulene (3%) were also present. Linalool, (-)-(5Z)- 2,6-dimethylocta-5,7-diene-2,3-diol, germacrene-D, β-selinene, (E)-3-hexenyl butyrate were found in low amounts ranging between 1-2.3%. Two chemotypes have been described from the Iberian Peninsula (Ariño et al. 1999); cis-epoxyocimene type and a cis-epoxyocimene + chrysanthenyl acetate type. The cultivated Spanish wormwood population VP used in this work showed cis-epoxyocimene + cischrysanthenol chemotype. Similarly, the population that originated this selected germplasm showed a similar VP composition (Bailén et al. 2013). The antiparasitic effects of the VP and its fractions are shown in Tables II, III. The VP was active on T. cruzi up to a concentration of 200 µg/mL, showing GI of 100% at 800 µg/mL, 96% at 400 µg/mL and 83.6% at 200 µg/ mL (GI50 144.6 µg/mL), in agreement with the bioactivity reported for the parent population EO (Bailén et al. 2013). This VP also showed a trichomonacidal effect (Table III) with GI of 99.1% at 500 µg/mL, 87.4% at 250 µg/mL and 53.7% at 100

µg/mL (GI50 87.2 µg/mL). Among the VP fractions, VLC1 and 2 were the most active on T. cruzi (nearly 100% mortality at 100 µg/mL) (Table II). Seven of the nine VLC fractions (1, 2, 4-8) showed trichomonacidal effect (> 70%) at 500 µg/mL, but only VLC1 maintained significant activity (81.6%) at 100 µg/mL. VLC4 and VLC8 were also active at 250 µg/mL (Table III).

TABLE I Gas chromatography mass spectrography analysis of the Artemisia absinthium vapour pressure (VP) essential oil (T2-11) and its fractions

Compound	EO	Fraction								
	AaT11ae	VLC1	VLC2	VLC3	VLC4	VLC5	VLC6	VLC7	VLC8	VLC9
Yield (%)	-	12.6	4	0.8	7.6	5.3	13.5	4.8	7.8	1.7
Linalool	2.0	-	-	-	-	-	-	-	17.3	-
cis-Epoxycimene	39.8	-	-	-	13.5	63.9	86.6	5.6	-	-
Camphor	4.5	-	-	-	-	1.4	7.2	27.0	-	-
Chrysanthenol	11.9	-	-	-	-	-	-	60.8	63.0	0.7
(*E*)-3-hexenyl butyrate	1.1	-	-	-	-	-	-	1.7	12.4	-
Chrysanthenyl acetate	5.3	-	-	1.0	35.7	22.1	0.9	-	-	-
(5Z)-2,6-dimethylocta-5,7-diene-2,3-diol	2.0	-	-	-	-	-	-	-	-	67.8
trans-Caryophyllene	3.8	29.5	-	-	-	-	-	-	-	-
Germacrene-D	2.3	15.5	-	-	-	-	-	-	-	-
β-Selinene	1.1	8.8	-	-	-	-	-	-	-	-
Dihydrochamazulene	5.8	2.5	42.5	7.9	-	-	-	-	-	-
Chamazulene	2.6	1.1	41.4	81.7	-	-	-	-	-	-

data are expressed as relative abundance (%).

TABLE II. Trypanocidal activity of Artemisia absinthium vapour pressure (VP) essential oil (T2-11), fractions VLC1-VLC9, trans-caryophyllene and (-)-cis-chrysanthenol

EO, fractions and compounds	Concentration					
	800 µg/µl	400 µg/µl	200 µg/µl	100 µg/µl	10 µg/µl	1µg/µl
AaT11Ae	100±1.7	96.0±11.2	83.6±18.0	33.0± 7.2	5.8± 5.2	4.7± 4.9
VLC1	-	-	-	97.8±4.6	0.6± 1.8	0.5± 1.1
VLC2	-	-	-	94.7±8.4	1.5± 2.3	1.3± 2.2
VLC3	-	-	-	30.0±5.4	0.9± 1.7	0.0± 0.0
VLC4	-	-	-	46.7± 15.3	2.0± 3.8	4.5± 4.2
VLC5	-	-	-	26.8± 15.9	1.0± 1.3	0.5± 1.8
VLC6	-	-	-	8.4± 6.4	1.6± 2.0	3.3± 4.3
VLC7	-	-	-	10.1±7.6	0.0± 0.0	4.8± 5.9
VLC8	-	-	-	41.9± 10.1	11.1± 5.6	4.5± 3.7
VLC9	-	-	-	6.5 ± 8.1	0.8 ± 1.4	1.4 ± 2.3
trans-caryophyllene (**8**)	-	-	-	99.4 ± 1,9	11.6 ± 14.1	5.4 ± 5.5
(-)-*cis*-chrysanthenol (**4**)	-	-	-	3.5 ± 8.3	0.0 ± 0.0	0.4 ± 0.7
NFX	-	-	-	99.9 ± 0.2	100.0 ± 0.5	20.8 ± 2.8

nifurtimox (NFX) is included as drug reference. Data are expressed as percentage of growth inhibition ± standard deviation.

Fractions VLC1 and 2 were not cytotoxic against nontumour cell line HS5, suggesting selective antiparasitic activity for these two fractions (Table IV). Fraction VLC1 is characterised by trans-caryophyllene (29.5%), germacrene D (15.5%) and β-selinene (8.8%). Fraction VLC2 contained dihydrochamazulene (42.5%) and chamazulene (41.4%). The main component of VLC8 is (-)-cis-chrysanthenol (63%) (Figure, Table I). trans-caryophyllene showed a remarkable activity against T. cruzi (Table II) with GI of 99.4% at 100 µg/mL (GI50 39.2 µg/mL). This compound also showed significant trichomonacidal effect (Table III) with GI of 100% at 500 µg/mL, 99% at 250 µg/mL, 80.3% at 100 µg/mL and 53.7% at 75 µg/mL (GI50 68.7 µg/mL). The antiparasitic effects of VLC1 against T. cruzi and T. vaginalis can be partially attributed to trans-caryophyllene. The activity of VLC2 on T. cruzi cannot be attributed to chamazulene since fraction VLC3, containing 81.7% chamazulene, was

inactive. Therefore, dihydrochamazulene or the mixture of both compounds could be responsible for this effect. Furthermore, neither VLC2 nor VLC3 showed trichomonacidal activity at 100 µg/mL, suggesting a selective activity (alone or in synergy) of dihydrochamazulene on T. cruzi. (-)-cis-chrysanthenol also showed activity against T. vaginalis with GI of 100% at 500 µg/mL, 96.3% at 250 µg/mL, 54.5% at 100 µg/mL and 45.4% at 75 µg/ mL (GI50 87.2 µg/mL) (Table III). The effects of VLC8 against T. vaginalis at high concentrations can be attributed to (-)-cis-chrysanthenol.

When tested against tumour cell lines, the VP and fractions inhibited the growth of human tumour cell lines in a dose-dependent manner (Table IV). We carried out parallel experiments with cisplatin and paclitaxel on cells plated at the same time from a single flask to serve as reference cytotoxicity. Doses used for these two compounds are those that lead to a 50% of GI in most tumour cell lines. For the fractions tested, the most sensitive cell lines were SK-MEL-5 (melanoma) and HCT116 (colorectal adenocarcinoma). MCF-7 (luminal breast adenocarcinoma) was the less sensitive tumour cell line. VP and VLC-1 were the most active fractions for the majority of cell lines, being also remarkable cytotoxic effects of VLC-5 and VLC-8 fractions. Moreover, VP and VLC-1 also showed activity against A549 and H292 cell lines (adenocarcinoma and squamous nonsmall cell lung cancer). These cytotoxic effects may be due to the presence of trans-caryophyllene and/or germacrene D in the VP and fractions. The cytotoxic activity of trans-caryophyllene on animal and human tumour cells has been previously described by other authors (el Hadri et al. 2010). It is noteworthy that these fractions had little or no cytotoxic effect against the nontumour cell line, HS5, suggesting selective cytotoxic activity for these fractions. This would be an important biological effect and could provide new rational basis for the design of new antitumour compounds. EOs rich in chamazulene showed variable growth-inhibitory effects on human cancer cell lines with GI50 values ranging from 14.3 µg/mL on A375 (human malignant melanoma cell line) to 59.8 µg/mL on T98G (human glioblastoma cell line) (Ornano et al. 2013) and strong antifungal properties against dermatophytes and opportunistic saprophytes (Jamalian et al. 2012). Chamazulene has been reported to be a potent hydroxyl radical scavenger capable to effectively inhibit lipid peroxidation (Siveen & Kuttan 2011, Ornano et al. 2013) and possess antiinflammatory activity in vivo, by inhibiting the leukotriene synthesis and lipid peroxidation (Safayhi et al. 1994, Rekka et al. 1996). However there are no reports on antiparasitic effects of chamazulene or dihydrochamazulene.

TABLE III Trichomonacidal activity of Artemisia absinthium vapour pressure (VP) essential oil (T2-11), fractions VLC1-VLC9, trans-caryophyllene and (-)-cis-chrysanthenol

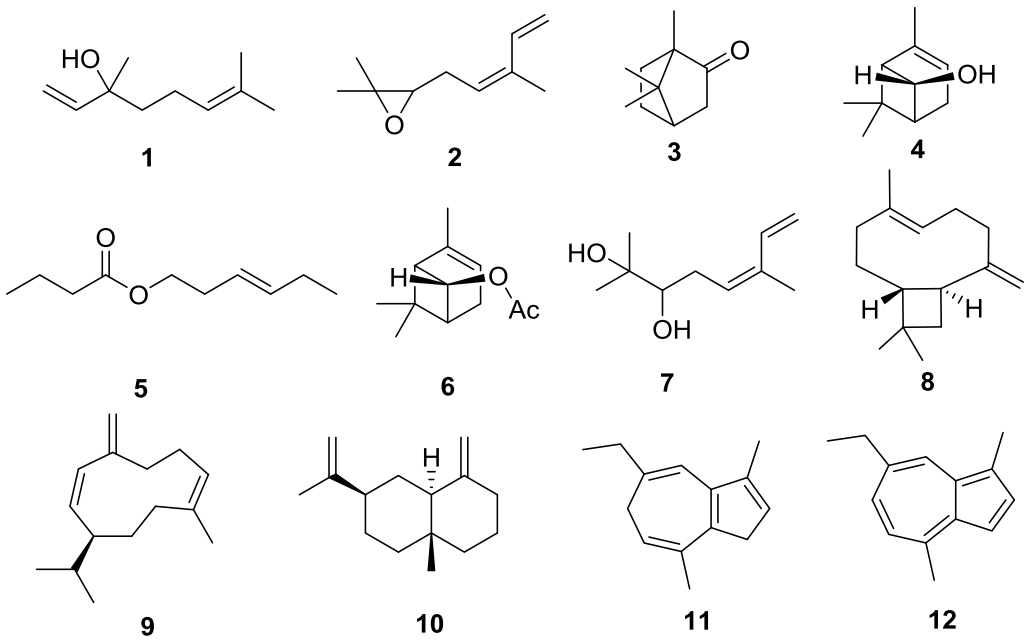
EO, fractions and compounds	Concentration					
	500 µg/ml	250 µg/ml	100 µg/ml	75 µg/ml	37.5 µg/ml	18.75 µg/ml
VP T2-11	99.1 ± 0.4	87.4 ± 1.3	53.7 ± 0.5	51.7 ± 3.3	14.5 ± 2.4	0.8 ± 1.2
VLC1	99.3 ± 0.2	98.8 ± 0.2	81.6 ± 0.2	47.6 ± 3.8	10.3 ± 2.0	0.4 ± 0.5
VLC2	100 ± 0.0	44.3 ± 5.6	11.8 ± 4.0	2.7 ± 3.9	1.2 ± 1.7	0.0 ± 0.0
VLC3	35.5 ± 0.1	12.2 ± 2.9	10.3 ± 2.0	0.4 ± 0.5	1.0 ± 1.4	0.0 ± 0.0
VLC4	99.5 ± 0.1	99.4 ± 0.1	39.1 ± 0.2	27.0 ± 3.2	4.0 ± 0.1	0.0 ± 0.0
VLC5	99.3 ± 0.4	43.3 ± 2.6	2.4 ± 3.4	3.7 ± 5.3	0.0 ± 0.0	0.0 ± 0.0
VLC6	71.6 ± 1.3	36.5 ± 1.8	4.7 ± 6.6	2.5 ± 3.6	0.4 ± 0.6	0.0 ± 0.0
VLC7	100.0 ± 0.0	47.9 ± 4.3	27.0 ± 6.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
VLC8	99.9 ± 0.6	92.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
VLC9	4.7 ± 6.7	1.7 ± 2.5	0.0 ± 0.0	1.7 ± 2.4	0.0 ± 0.0	0.5 ± 0.7
trans-caryophyllene (**8**)	100.0 ± 0.0	99.0 ± 0.2	80.3 ± 7.3	53.7 ± 2.2	34.3 ± 6.0	19.9 ± 8.5
(-)-*cis*-chrysanthenol (**4**)	100.0 ± 0.0	96.3 ± 1.4	54.5 ± 5.9	45.4 ± 2.5	40.9 ± 0.8	2.1 ± 0.8

data are expressed as percentage of growth inhibition ± standard deviation

TABLE IV Cytotoxic effects on A549, H292, HCT116, MCF7, SK-MEL-5 and HS5 cells of Artemisia absinthium vapour pressure (VP) essential oil (T2-11) and fractions VLC1-VLC9

		Cell lines					
EO and fractions	Concentration	A549	H292	HCT116	MCF7	SKMEL-5	HS5
VP T2-11	250 µg/ml	79.5±0.4	76.7±1.1	51.1±1.8	91.3±0.9	60.1±3.1	80.5±3.4
	100 µg/ml	98.6±5.2	82.5±1.0	58.5±3.5	95.7±1.8	78.5±0.9	86.7±0.9
VLC-1	250 µg/ml	60.6±1.5	73.0±0.9	49.6±1.3	95.7±1.8	58.2±0.8	80.8±1.5
	100 µg/ml	74.8±1.9	77.2±3.2	52.1±0.8	91.3±0.9	56.9±3.0	79.1±2.4
VLC-2	250 µg/ml	84.0±4.7	89.2±7.8	59.4±4.2	84.0±9.8	58.8±10.5	91.7±6.8
	100 µg/ml	69.5±10.2	90.4±2.0	59.5±3.6	89.6±2.1	67.7±5.2	83.3±7.4
VLC-3	250 µg/ml	83.6±4.2	97.2±7.1	59.0±1.4	91.9±3.5	57.3±5.7	90.2±5.2
	100 µg/ml	100±9.1	98.1±5.4	63.4±0.9	93.3±0.5	66.3±5.5	84.1±4.3
VLC-4	250 µg/ml	80.6±2.5	73.5±3.1	63.9±1.3	88.7±0.2	57.4±1.0	78.5±2.5
	100 µg/ml	95.9±2.3	79.9±0.8	78.5±0.6	100±0.7	58.8±1.6	86.1±1.8
VLC-5	250 µg/ml	57.7±1.3	78.1±0.6	51.7±1.5	80.1±3.7	54.1±2.1	75.9±1.7
	100 µg/ml	95.2±3.1	79.7±1.3	62.8±3.6	97.3±2.3	53.5±3.9	68.2±1.8
VLC-6	250 µg/ml	100±2.5	87.8±3.9	64.4±2.2	96.9±2.3	59.5±4.2	81.2±2.9
	100 µg/ml	100±2.7	96.8±1.8	75.0±2.7	100±1.1	72.0±1.3	90.7±3.7
VLC-7	250 µg/ml	100±3.4	94.4±1.1	93.1±1.9	93.7±1.6	69.5±8.9	89.1±1.8
	100 µg/ml	100±2.3	100±1.7	93.6±2.3	97.9±1.9	78.4±5.1	95.7±2.1
VLC-8	250 µg/ml	67.7±4.6	82.8±13.6	50.9±2.0	69.1±2.3	54.5±1.9	76.3±3.9
	100 µg/ml	100±2.4	87.2±5.2	65.0±0.6	100±1.7	54.5±2.8	86.6±2.8
VLC-9	250 µg/ml	100±0.2	92.5±6.0	96.8±1.6	97.8±1.7	65.9±6.0	95.6±1.7
	100 µg/ml	100±3.1	97.4±5.0	89.6±3.5	96.1±1.7	79.3±2.1	95.4±2.1
Paclitaxel	100 µM	41.9±2.9	41.8±1.7	40.2±2.8	52.9±3.5	34.9±2.4	27.7±4.8
Cisplatin	1 mM	34.6±1.5	57.3±2.6	25.7±1.7	65.6±1.1	42.6±0.3	22.8±2.4

paclitaxel and cisplatin are included as drugs reference. Data are expressed as viability percentage ± standard deviation relative to vehicle-treated control cells.



Chemical structures of linalool (1), cis-epoxycimene (2), camphor (3), (-)-cis-chrysanthenol (4), (E)-3-hexenyl butyrate (5), chrysanthenyl acetate (6), (-)-(5Z)-2,6-dimethylocta-5,7-diene-2,3-diol (7), transcaryophyllene (8), germacrene-D (9), β-selinene (10), 3,6-dihydrochamazulene (11) and chamazulene (12).

trans-caryophyllene has been reported as antinociceptive (Katsuyama et al. 2013), anxiolytic, antidepressant (Bahi et al. 2014) and antimicrobial (Goren et al. 2011), among other effects. EOs rich in *trans*-caryophyllene and caryophyllene oxide from different plant species have been tested on T. cruzi, T. vaginalis (Vermani & Garg 2002, Cheikh-Ali et al. 2011, Polanco-Hernández et al. 2012, 2013, Costa et al. 2013, da Silva et al. 2013, Monzote et al. 2014) and other protozoan parasites such as *Trypanosoma brucei*, *Plasmodium falciparum* and *L. infantum* (Nibret & Wink 2010, Gachet et al. 2011, Monzote et al. 2014), with different activity ranges alone or showing synergistic effects with the different EO components (Setzer et al. 2007, Polanco-Hernández et al. 2012, 2013). In our study, *trans*-caryophyllene was effective against *T. cruzi* and *T. vaginalis*, but the activity of fraction VLC1 is not fully explained by its content in *trans*-caryophyllene, suggesting synergistic effects. In summary, the most interesting compounds are trans-caryophyllene (main compound in fraction VLC1), for its demonstrated antiparasitic and cytotoxic activity and 3,6-dihydrochamazulene, because of the activity of fraction VLC2; both compounds present in the A. absinthium var. candial® VP EO.

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4.6. Comparative chemistry and insect antifeedant effects of conventional (Clevenger and Soxhlet) and supercritical extracts (CO_2) of two *Lavandula luisieri* populations

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Industrial Crops and Products (**2014**), 58, 25-30.
doi:10.1016/j.indcrop.2014.03.021

Abstract

The objective of this study was the preparation of supercritical (SCE), conventional hydrodistilled (HDE) and organic solvent (OSE) extracts from two Iberian *Lavandula luisieri* populations (A and B) to study their chemical composition and their insect antifeedant properties against *Spodoptera littoralis* and *Myzus persicae*. HDE and SCE extracts had different chemical compositions and insect antifeedant effects, extracts from population A being more effective. Compounds 5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one (**6**) and (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate (**7**)were the major components of the SCE fractions of both populations. The supercritical technique (SFE) improved the extraction of **6**, **7**, oxo-cadinol (**8**), hexadecanoic acid and several not identified compounds with respect to HDE. SCE extracts also exhibited increased antifeedant effects against *S. littoralis* and the aphid *M. persicae* (A-SCE). The biological effects of the active extracts cannot be accounted for those of their individual major components, suggesting additive or synergistic effects.

***Key Words**: Lavandula luisieri;* Supercritical fluid extraction; Antifeedant activity; Essential oils; Necrodane-type compounds

1. Introduction

Lavandula luisieri (Rozeira) Riv.-Mart. (Rivas-Martínez, 1979, Rivas-Martínez et al., 2002a and Rivas-Martínez et al., 2002b) is a small aromatic shrub endemic to the Iberian Peninsula and common in the semi-arid regions of Southern Portugal and Southwest Spain. Previous studies showed that *L. luisieri* oil contained 1,8-cineole, lavandulol, linalool and their acetates, also present in other *Lavandula* species, in addition to a series of compounds with a 1,2,2,3,4-pentamethylcyclopentane (necrodane) structure (Baldovini et al., 2005, García-Vallejo et al., 1994 and Lavoine-Hanneguelle and Casabianca, 2004). Necrodane derivatives have previously been found in the defensive secretions of the beetle *Necrodes surinamensis* (Eisner et al., 1986 and Roach et al., 1990), suggesting a potential defensive role played by these compounds. Furthermore, *trans*-α-necrodyl isobutyrate has been found to be a sex pheromone of the grape mealybug *Pseudococcus maritimus* (Figadere et al., 2007). *L. luisieri* essential oil and extract has also proven to be active against *Candida albicans* and Gram positive bacteria (Baldovini et al., 2005).

The chemotype distribution and bioactivity of *L. luisieri* essential oil in the Iberian Peninsula has been studied. Samples from central and southern *L. luisieri* populations exhibited a wide variation in terms of their yield and composition. Their major components were camphor, 1,8-cineole and 2,3,4,4-tetramethyl-5-methylen-2-cyclopenten-1-one (**2**) (Sanz et al., 2004). The essential oils from these plant groups were active against the aphid *Myzus persicae* (González-Coloma et al., 2006). Additionally, the volatile composition of western *L. luisieri* populations showed that their major compound was *trans*-α-necrodyl acetate with less variability in their composition (González-Coloma et al., 2011).

L. luisieri may be a viable alternative crop for the production of bioactive compounds in the Iberian Peninsula. A preliminary short-term experimental cultivation (2 years) showed that the essential oils maintained or improved their insect antifeedant effects with respect to the wild population except for the ethanolic extracts (González-Coloma et al., 2011). Compounds with a necrodane skeleton and other secondary metabolites including oxo-cadinane, rosmarinic acid and olenanane type triterpenes (β-amyrin and the acids tormentic, ursolic and oleanolic,) have been isolated from *L. luisieri* aerial parts (González-Coloma et al., 2011, unpublished).

As part of our ongoing search for botanical biopesticides, the chemical composition of *L. luisieri* hydrodistillation (HDE), Sohxlet-organic solvent (OSE) and CO_2 supercritical (SCE) extracts have been analyzed in this study using GC–MS and HPLC–MS and their insect antifeedant effects tested against *Spodoptera littoralis* and *M. persicae*. Additionally, we tested the insect antifeedant activity of the major compounds from *L. luisieri*.

2. Experimental

2.1. Plant material

Flowering plants were collected in June 2007 from a cultivated population of *L. luisieri* (A) previously described (see González-Coloma et al., 2011) and a wild population (B) located in Pueblo Nuevo del Bullaque (Ciudad Real, Spain, latitude: 39°27′41″ N, longitude: 4°24′34″ W, altitude: 733 m).

2.2. SCE, HDE and OSE extract preparation

The SFE plant and extraction procedure was previously described (Langa et al., 2009 and Martín et al., 2011). The extract collected in Separator 1 was collected at the end of the experiment (W), while several fractions were collected in Separator 2 during the experiment (F*i*; *i* = 1, 2, 3). The extraction conditions with CO_2 were 135 bar, 40 °C, 10.5 L/min and particle size 0.450 mm (SCE1). An extra SFE experiment was conducted on previously extracted plant material (180 bar, 40 °C, 10.5 L/min) with the addition of 50 mL of ethanol (EtOH) as a co-solvent (SCE2). SFE was performed on 100 g plant samples and the average yields were 1.3% (F*i* = 1.0% and W = 0.3%) and 1.4% (F*i* = 1.3% and W = 0.1%) for populations A and B, respectively. Representative samples of different SFE experiments were selected to analyze both composition and activity. The experimental conditions of the selected experiments are shown in Table 1.

Table 1. Supercritical experiments, plant material used and extraction conditions.

Population	Experiment	Fluid	*P* (MPa)	*T* (ºC)	ρ_{sc} (kg/m^3)	Flow rate (L/min)
A	SCE1	CO_2	13.5	40	753.6	10.5
	SCE2	CO_2 + EtOH	18.0	40	819.5	10.5
B	SCE1	CO_2	13.5	40	753.6	10.5
	SCE2	CO_2 + EtOH	18.0	40	819.5	10.5

HDE was performed on 100 g plant samples in a Clevenger-type apparatus (0.4% yield for A and B) according to the method recommended by European Pharmacopoeia (http://www.edqm.eu/en/Homepage-628.html).

OSE was performed in a Soxhlet apparatus with EtOH and concentrated in vacuo (18 and 12% yield for A and B, respectively).

2.3. Essential oil analysis

Leaf essential oils were analyzed by GC–MS using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, California, USA) coupled to an Agilent 5973N mass detector (electron ionization, 70 eV) (Agilent Technologies, Palo Alto, California, USA) and equipped with a 25 m × 0.2 mm id HP-1 (methyl polisiloxane, 0.2 µm film thickness) and a 30 m × 0.25 mm id Carbowax (polyethylene glycol, 0.25 µm film thickness) capillary columns (Hewlett-Packard). Working conditions were as follows: injector temperature, 260 °C; temperature of the transfer line connected to the mass spectrometer, 280 °C; column temperature 70–190 °C, 5 °C min^{-1}. EI mass spectra and retention data were used to identify compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (Mc Laferty and Stauffe, 1989). Quantitative data were obtained from the TIC peak areas without the use of response factors.

2.4. HPLC–MS analysis

The OSEs were analyzed by HPLC–MS on a Shimadzu LC-20AD HPLC coupled to a LCMS-2020 QP mass spectrometer using an electrospray ionization (ESI) interface and a Teknokroma, Mediterranea Sea_{18} column (250 mm × 4.6 mm, 5 µm particle size) with an ACE 3 C18 analytical guard cartridge. The compounds were eluted with methanol (MeOH): 0.1% acetic acid in milli-Q water 38:100% gradient for 45 min, 100% MeOH for 10 min and 100:38% for 13 min at 0.5 mL/min and 15 L/min nitrogen (drying gas for solvent evaporation) flow rates. The electrospray capillary potential was set to +4.50 kV and ESI was conducted in the Full Scan positive mode (*m*/*z* = 145–545) with a potential of 1.30 kV and a capillary temperature of 250 °C. Stock solutions of extracts (0.25 µg/µL), compounds 1, 5, 8 (isolated from *L. luisieri*; González-Coloma et al., unpublished) and oleanolic acid (Sigma) (0.05 µg/µL) were dissolved in MeOH for sample injection (10 µL). All the solvents used were HPLC–MS grade (Fig. 1).

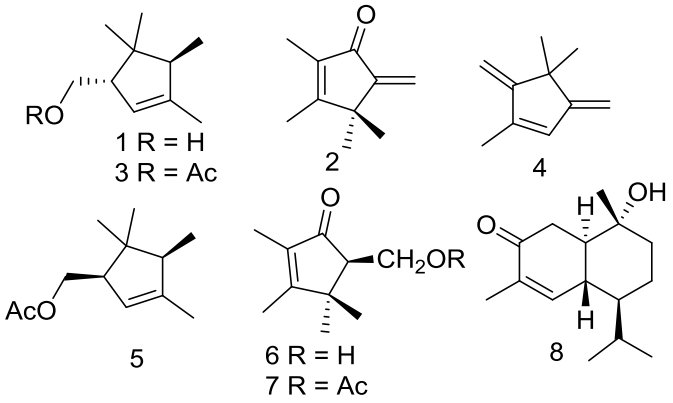

Figure 1. Chemical strucures of compounds **1**-**8**.

2.5. Insect bioassays

S. littoralis and *M. persicae* colonies were reared on artificial diet and bell pepper (*Capsicum annuum*) plants respectively, and maintained at 22 ± 1 °C, >70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber. Bioassays were conducted with newly emerged *S. littoralis* L6 larvae or ten *M. persicae* adults as described by Burgueño-Tapia et al. (2008).

3. Results and discussion

3.1. HDE, SCE AND OSE composition

Several extracts were obtained from two populations of *L. luisieri* (A and B), including hydrodistilled (HDE), ethanolic (OSE) and supercritical (SCE). The chemical composition of these extracts is shown in Tables 2 (GC–MS) and 3 (LC–MS).

Table 2. Chemical composition (% relative) of the HDE and SCE extracts from populations A and B, analyzed by GC–MS.

Compounds	Population and extract														
	A								B						
	HDE	SCE1			SCE2				HDE	SCE1			SCE2		
		F1	F2	W	F1	F2	F3	W		F1	F2	W	F1	F2	F3	W
α-Pinene	1.2															
1,8-Cineole	4.8															
Fenchone									5.2							
Linalool	1.4															4.5
Camphor									74.4	7.4						
trans-α-Necrodol (**1**)	7.7															
cis α-Necrodol	3.3															
2,3,4,4-Tetramethyl-5-methylidenecyclopent-2-en-1-one (**2**)	2.0	2.7	8.0	12.2	2.3	3.9	6.7	4.6	5.3	4.2	2.4			3.9	3.8	5.9
cis-Linalool oxide	3.2															
trans-α-Necrodyl acetate (**3**)	33.0		1.7						8.2	2.3						
Geranyl acetate	14.2															
3,5-Dimethylene-1,4,4-trimethylcyclopentene (**4**)									2.8							
cis-α-Necrodyl acetate (**5**)	5.9															6.3
5-Hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one (**6**)		11.6	16.0	3.5	8.5	10.8	9.4	5.1		11.9	9.3	7.8	3.4	7.9	8.5	5.9
$C_9H_{10}O_3$		8.2	2.5	4.5	3.0	2.5										
Not identified (43 86 153 83 41)		4.7	4.5	5.7	3.6	4.5	3.9			3.7	7.9	3.9	6.7	5.9	6.0	3.7
Not identified (43 86 153 83 41)											9.6	4.7	4.4	5.1	6.3	2.4
(2,2,3,4-Tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate (**7**)		12.1	29.2	31.8	20.9	22.9	17.4	5.7		38.1	18.1	15.7	15.8	12.1	14.1	17.4
β- Selinene	4.3	5.3	5.3	6.3	6.8	7.7	7.0				7.0					
$C_{15}H_{24}$									2.8	12.3						
$C_{15}H_{24}$									1.2	3.9		4.7	6.4	5.2	6.1	2.2
3-Oxo-cadinol (**8**)			2.9		3.5	4.1	3.9	7.5		7.4		16.8	21.4	15.3	21.9	22.1
$C_{15}H_{24}O_2$	3.8															
$C_{15}H_{24}O_2$	3.5															
$C_{15}H_{26}O_2$										1.9		11.9	11.6	8.9	9.3	23.3
$C_{18}H_{28}O_3$		8.2	2.1		3.4	4.8	3.2	5.5								
Hexadecanoic Acid		3.1	5.6	6.0	14.5	2.2	9.0	11.4				5.2				
Not identified (43 135 161 109 189)		3.2									17.8					

The HDEs showed quantitative and qualitative variations in their composition (Table 2). Elemental composition is given in Table 2 for components whose mass spectral fragmentation allowed obtain information about their molecular weight and structural characteristics.

The major components present in A-HDE were *trans*-α-necrodyl acetate (**3**), geranyl acetate, *trans*-α-necrodol (**1**) and *cis*-α-necrodyl acetate (**5**). The HDE composition reported previously for the same *L. luisieri* population was similar (González-Coloma et al., 2011). The major component of the B-HDE was camphor, followed by **3**, 2,3,4,4-tetramethyl-5-methylenecyclopent-2-en-1-one (**2**) and fenchone. A similar composition was previously reported for leaves and flowers of another *L. luisieri* population from the same geographical area (Toledo group 5 in González-Coloma et al., 2006). The qualitative differences between HDEs A and B involved linalool, *trans*- and *cis*-α-necrodol (**1**), unidentified ($C_{10}H_{18}O_2$), geranyl acetate, **5**, β-selinene and two unidentified ($C_{15}H_{24}O_2$) in population A, plus fenchone, camphor and two unidentified ($C_{15}H_{24}$) in population B (Table 2).

5-Hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one (**6**) and (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate (**7**) were the major components of the SCE fractions of both populations. The supercritical technique (SFE) improved the extraction of **6**, **7**, oxo-cadinol (**8**) in A and B fractions; $C_{18}H_{28}O_3$ and hexadecanoic acid in A and β-selinene, $C_{15}H_{24}$, $C_{15}H_{26}O_2$ in B, when compared to the HDE. The chemical composition of A and B fractions was different due to the presence of $C_9H_{10}O_3$ (A1 and A2.F1, A2.F2), and $C_{18}H_{28}O_3$ (A1–A2) in the A samples. Linalool (B2.W), camphor (B1.F1), *cis*-α-necrodyl acetate (**5**) (B2.W), two $C_{15}H_{24}$ (B1.F1 and B1.F1, B1.W-B2) and one $C_{15}H_{26}O_2$ (B1.F1, B1.W-B2) were found in the B fractions. In addition to these qualitative differences between A and B there were also some quantitative ones. Compound **8** was more abundant in B samples and hexadecanoic acid was more abundant in the A ones (Table 2).

The HPLC–MS analysis of the OSE extracts showed qualitative and quantitative differences (Table 3). Both extracts contained compound **6**, rosmarinic acid, tormentic acid, and oleanolic acid. The A-OSE extract showed unidentified parent ions of *m*/*z* 525, 385/351, plus ursolic acid. B-OSE showed unidentified parent ions of *m*/*z* 449, 383, 463, 259, 493 and Compound **8**. These compounds were previously isolated from *L. luisieri* aerial parts (González-Coloma et al., unpublished).

Table 3. Chemical composition (% relative) of the OSE extracts analyzed by HPLC-MS.

Parent Ions (*m/z*)	MW (*m/z*)	RT (min)	Population		Identified compound
			A %	B %	
191 [M+Na]+	168	26.35	7.93	4.33	5-Hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one (**6**)
383 $[M+Na]^+$	360	27.82	3.41	7.32	Rosmarinic acid
259 $[M+Na]^+$	236	45.05	-	10.03	3-Oxo-cadinol (**8**)
511 $[M+Na]^+$	488	50.77	-	13.85	Tormentic acid / isomer
		51.48	10.90	1.99	
479 [M+Na]+	456	61.62	3.47	2.95	Oleanolic acid
479 [M+Na]+	456	62.12	12.4	-	Ursolic acid

3.2. Antifeedant and insecticidal activity of HDE OSE and SCE

Table 4 shows the bioactivity of the extracts on the target species. A-HDE was a strong antifeedant against *S. littoralis* and B-HDE had a moderate-low effect. Similarly, HDEs from this population have been reported as being efficient antifeedants to *S. littoralis* but exhibiting only a moderate-low effect on *M. persicae* (González-Coloma et al., 2011); an HDE from a population similar to B was also inactive against these insects (González-Coloma et al., 2006). Both OSE extracts were inactive against *M. persicae* but A had a moderate-low antifeedant effect on *S. littoralis*. Overall, the A-SCE extracts were strong *S. littoralis* antifeedants with activity potencies within the range or stronger (SC1.F1, SC2.F3) than A-HDE. When tested against *M. persicae*, all the A-SCE fractions (except 2.1) were strong aphid deterrents in contrast to the inactive A-HDE and A-OSE.

Table 4. Antifeedant and insecticidal activity of the different *L. luisieri* extracts against *S. littoralis* and *M. persicae.*

Extraction and fraction		Population			
		A		B	
		***S. littoralis*[a]**	***M. persicae*[b]**	***S. littoralis*[a]**	***M. persicae*[b]**
HDE		97.0 ± 1.0* 10.23 [c] (7.06-14.78)	31.4 ± 5.2	67.1 ± 8.2	40.8 ± 7.6
OSE		66.0 ± 4.0	55.1 ± 7.9	29.0 ± 11.5	50.5 ± 6.5
SCE1	**1**	86.3 ± 5.1 5.63 [c] (2.13-14.85)	99.4 ± 5.5* 19.42 [c] (13.48-20.00)	32.3 ± 12.6	58.6 ± 4.5*
	2	66.7± 6.5	97.5 ± 3.4* 30.45 [c] (25.99-35.65)	59.7 ± 11.2*	39.2 ± 6.5
	W	96.6 ± 14.2* nc	91.1 ± 3.1* nc	63.3 ± 7.5*	55.9 ± 5.7*
SCE2	**1**	89.0 ± 6.7* 32.13 [c] (22.04-46.82)	60.2 ± 5.3*	72.7 ± 8.5*	51.2 ± 7.7
	2	69.6± 6.5	94.4 ± 4.5* 20.0 [c] (14.10-28.29)	84.7 ± 5.9* 37.4 [c] (26.9-51.7)	63.3 ± 7.7*
	3	86.2± 3.6 5.55 [c] (3.83-8.03)	94.7 ± 4.1* 19.0 [c] (13.98-25.80)	84.3±4.9 10.98 [c] (4.72-25.62)	51.2 ± 8.7
	W	69.8 ± 7.7	97.3 ± 6.2* 32.0 [c] (18.59-55.09)	69.0 ± 5.0*	57.8 ± 7.6*

[a]Percent settling inhibition. [b]Percent feeding inhibition. (%FI / %SI = [(1-(T/C))*100] where T = consumption /settling on treated disk; and C = consumption /settling on Control disk.

*p<0.05, Wilcoxon Paired Rank Test.

[c]EC_{50} (effcient dose to give a 50 % feeding / settling inhibition, µg/cm^2) and 95 % confidence limits (lower, upper).

Nc, not enough sample to calculate EC_{50}

In general, the B-SCE extracts had lower insect antifeedant effects than the A-SCE ones as shown for their HDEs. Fractions B-SCE2-F1 and 2-F2 were antifeedants against *S. littoralis* in contrast to the inactive B-HDE and B-OSE. The antifeedant effects of the BSC fractions on *M. persicae* were low-moderate but were generally stronger than the HDE (except for B1.F2).

These results indicate that supercritical extraction of *L. luisieri* enhanced the antifeedant effects of A and B2 (with the addition of EtOH to the SFE extraction process) extracts against *S. littoralis* and *M. persicae* and when compared to the HDEs and OSEs. Similar results were obtained for the insect antifeedant effects of SCE extracts from *Artemisia absinthium* (Martín et al., 2011).

Table 5 shows the activity of the major pure compounds present in *L. luisieri*. Compounds**2**, **6** and **8** were antifeedant against *S. littoralis*. A mixture of isomers **3** and **5** acted as a moderate-low antifeedant againsty *M. persicae*, while camphor was not active against the insect species targeted here as previously shown (González-Coloma et al., 2006). None of the compounds on their own accounted for the activity of the extracts.

Table 5. Antifeedant and insecticidal activity of compounds isolated from *Lavandula luisieri*

Product	*S. littoralis*	*M. persicae*
	(%FI)[a]	(%SI)[b]
Fenchone	27.16±5.1	48.5±8.4
Camphor	22.64±6.0	37.6±7.0
2,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one (**2**)	72,3±15,5	49,7±11,3
Trans + *cis*-α-necrodyl acetate (**3+5**)	9.2±9.2	62.0±8.7
Hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one (**6**)	68.48±10.9	43.8±10.7
Rosmarinic acid	61.26±12.8	40.4±7.8
3-Oxo-Cadinol (**8**)	71,00±8,8	33,93±7,9
Tormentic acid	15.56±7.6	nc
Ursolic acid	23.71±15.2	31.39±9.8

[a]Percent settling inhibition. [b]Percent feeding inhibition. (%FI / %SI = [(1-(T/C))*100] where T = consumption /settling on treated disk; and C = consumption /settling on Control disk.
*p<0.05, Wilcoxon Paired Rank Test.
nc, not enough sample

In addition to these compounds, other known active components were present in the different extracts. Fenchone (present in the HDEs) exhibited acaricidal effects (Lee, 2004), moderate repellency to *Aedes aegypti* (Kim et al., 2002), was toxic to insects (Abdelgaleil et al., 2009, Kim and Ahn, 2001, Kim et al., 2002 and Nukenine et al., 2010) and inhibited AChE (Abdelgaleil et al., 2009). *M. persicae* exhibited a moderately significant response to fenchone. (González-Coloma et al., 2006). The repellent properties of linalool on various crop-destroying insects have been well documented (Kamatou and Viljoen, 2008, Kim and Lee, 2014, Koul et al., 2013, Mbata and Payton, 2013, Piesik et al., 2010 and Reisenman et al., 2013) including a moderate effect on *M. persicae* settling behavior (Rodilla et al., 2008). It also repels urban pests and insect vectors (Alzogaray et al., 2013 and Tarelli et al., 2009). α- and β-necrodol, present in A-HDE have proven to

repel ants and other insects and are irritants to cockroaches and flies (Eisner et al., 1986). Geranyl acetate (A-HDE) has been reported as a repellent and is toxic to mosquitoes (Michaelakis et al., 2014 and Samarasekera et al., 2006), is acaricidal (Samarasekera et al., 2006 and Ando, 1994) and is a strong aphid repellent (Birkett et al., 2010). Resins from the tropical legume Hymenaea, mostly composed of β-selinene (found in A-HDE, A-SCE, B-SCE1-2) have insect toxic and repellent properties (Langenheim et al., 1978). Fatty acids (Castillo et al., 2010) and their methyl esters (Santana et al., 2012) are involved in plant defense against insects. Specifically, hexadecanoic acid methyl ester proved toxic (Antonious et al., 2007 and Farghaly et al., 2009) and had an antifeedant effect on insects including *M. persicae* but not in the acid form (Santana et al., 2012).

The activity of *L. luisieri* A and B extracts could be explained by the additive or synergistic effects of their components. Similarly, the antifeedant effects observed for *L. luisieri*essential oils were not accounted for by their components individually (González-Coloma et al., 2006). *Salvia lavandulifolia* oil inhibits the enzyme acetylcholinesterase via a complex interaction between its constituents, including both synergistic and antagonistic interactions between the terpenes, with 1,8-cineole and camphor being very active components. However, a combination of camphor and cineol was antagonistic (Savelev et al., 2003). Thymol and linalool were synergistically toxic against *Helicoverpa armigera*, *Spodoptera litura* and *Chilo partellus* (Koul et al., 2013). Synergistic effects have been suggested for the insecticidal activities of SCEs from *A. absinthium* (Martín et al., 2011) and synergistic nematicidal effects have been found for several HDE components (Andres et al., 2012).

In conclusion, HDE, OSE and SCE extracts of *L. luisieri* plants from two populations (A and B) had different chemical compositions and insect antifeedant effects, the extracts from population A being more effective. The supercritical technique (SFE) improved the extraction of **6**, **7**, oxo-cadinol (**8**), hexadecanoic acid and several not identified compounds with respect to HDE. SCE extracts also exhibited increased antifeedant effects against *S. littoralis* and the aphid *M. persicae*. The biological effects of the active extracts cannot be accounted for those of their individual major components, suggesting additive or synergistic effects.

Acknowledgment

This work has been supported by grants CTQ2012-38219-C03-01 and CTQ2012-38219-C03-02 (MINECO-FEDER, Spain). Luis F. Julio and L. Martín gratefully acknowledge their JAE-CSIC and FPU-MICINN (AP2006-02054) predoctoral fellowships. S. Carlin is acknowledged for language revision.

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4.7. Phytotoxic and nematicidal components of Lavandula luisieri

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Journal of Natural Products (**2016**).
Manuscript under revision.

Several extracts were obtained from the aerial parts of pre-domesticated *Lavandula luisieri*, including essential oil (EO), ethanolic (EtOH), hexane (H) and ethyl acetate (EtOAc). Additionally, pilot plant vapor pressure extraction was carried out at a pressure range of 0.5-1.0 bar to give vapour pressure oil (PEO) and the hydrolate (WR) residue. The chemical study of the H extract led to the isolation of six necrodane derivatives (**1**, **2**, **4**-**7**), four of them (**1**, **2**, **5** and **7)** being described here for the first time, camphor (**9**), one cadinane sesquiterpene (**10**) and the triterpene acids tormentic (**11**) and ursolic (**12**). The EtOAc and EtOH extracts contained a mixture of polyphenols with rosmarinic acid (**13**) being the major component. Workup of the aqueous WR residue resulted in the isolation of **3** (necrodane derivative) and (1R*,2S*,4R*)-p-menth-5-ene-1,2,8-triol (**8**), both new natural compounds. The structures of these compounds were established based on their spectroscopic data. The role of these compounds in the phytotoxic and nematicidal activities of the extracts has been evaluated.

INTRODUCTION

Lavandula luisieri (Rozeira) Riv.-Mart.(Rivas-Martínez, 1979) is a small aromatic shrub endemic to the Iberian Peninsula and common in the semi-arid regions of Southern Portugal and Southwest Spain. Previous studies showed that *L. luisieri* oil contained 1,8-cineole, lavandulol, linalool and their acetates, also present in other *Lavandula* species, in addition to a series of compounds with a 1,2,2,3,4-pentamethylcyclopentane (necrodane) structure.(García-Vallejo *et al.*, 1994; Lavoine-Hanneguelle and Casabianca, 2004; Baldovini *et al.*, 2005a) *L. luisieri* essential oil has also proven to be antifungal, antibacterial and antioxidant,(Lavoine-Hanneguelle and Casabianca, 2004; Matos *et al.*, 2009; Roller *et al.*, 2009; Zuzarte *et al.*, 2012; Baptista *et al.*, 2015) and inhibits β-secretase (BACE-1), a promising therapeutic alternative for Alzheimer's disease,(Videira *et al.*, 2013) due to its content in 2,3,4,4,-tetramethyl-5-methylenecyclopent-2-enone.(Videira *et al.*, 2014)

The chemotype distribution and bioactivity of essential oils from *L. luisieri* exhibited a wide variation in the Iberian Peninsula. The major components were camphor, 1,8-cineole and 2,3,4,4-tetramethyl-5-methylen-2-cyclopenten-1-one for central and southern populations (Sanz *et al.*, 2004), and *trans*-α-necrodyl acetate for the western ones. These essential oils showed variable (moderate-strong) insect antifeedant effects.(González-Coloma *et al.*, 2006; González-Coloma *et al.*, 2011a) A preliminary short-term experimental cultivation of *L. luisieri* showed that the essential oils of cultivated plants maintained or improved their insect antifeedant effects with respect to the wild population.(González-Coloma *et al.*, 2011b) Additionally, the supercritical extraction (SCE) of *L. luisieri* improved the concentration of necrodane-type ketones, among other components with respect to hydrodistillation. SCE extracts also exhibited stronger insect antifeedant effects than the essential oil and ethanolic extract.(Julio *et al.*, 2014) Based on these results, we have initiated the domestication of *L. luisieri* (central Iberian Peninsula population) to obtain a viable variety for natural product based biopesticide production.

In this work we report on the chemical composition and biocidal effects (insect antifeedant, nematicidal and phytotoxic effects) of the essential oil (EO) and organic (ethanol, EtOH; hexane, H and ethyl acetate, EtOAc) extracts of experimentally cultivated *L. luisieri*. Additionally, pilot plant vapour-pressure extraction was carried out to give vapour pressure essential oil (PEO) and hydrolate (WR). Compounds with necrodane skeleton and other secondary metabolites from the bioactive extracts have been identified and their biocidal effects (insect antifeedant, phytotoxic and nematicidal effects) tested. Necrodane derivatives **1**-**3**, **5** and **7** and compound **8** are new natural products.

RESULTS AND DISCUSSION

Table 1 shows the phytotoxic effects of the different *L. luisieri* extracts. The aqueous WR extract strongly inhibited the germination of *L. sativa* and *L. perenne* at all the doses tested (100% inhibition at 100%, 50% and 25% WR solutions). The organic extracts were tested at two doses (0.4 and 0.20 mg/mL) but their effects on growth and germination at the lower dose (at 168h) were not significant (data not shown). All the organic extracts reduced *L. sativa* germination at 24h (97-54% reduction, both doses tested), being H and EtOH also effective at the end of the experiment (80% and 78% reduction at 0.40 mg/mL respectively). *L. sativa* plants grown after being treated with EO and EtOAc extracts showed a reduced root growth (70% and 49% reduction respectively). Overall the effects of

the organic extracts on *L. perenne* were lower, with a moderate effect on germination at 72h (36-78% reduction for both doses tested) and plant growth (30-40% reduction except for WROE).

Table 1. Phytotoxic effects of *L. luisieri* essential oil (EO), hexane (H), ethyl acetate (EtOAc), ethanolic (EtOH), hydrolate (WR) and its organic fraction (WROE) extracts.

Extract	mg/mL	*Lactuca sativa*			*Lolium perenne*			
		Germination		Growth	Germination		Growth (n)	
		24 h	144 h	root	72 h	168 h	leaf	root
EO	0.40	46.4 ± 13.1*	100 ± 0.0	29.4 ± 2.3*	50.0 ± 18.1*	100 ± 0	68.7 ± 7.0*	59.6 ± 5.0*
H	0.40	0*	20.0 ± 4.1*	na	64.0 ± 20.6*	84.6 ± 5.4	82.0 ± 11.3	67.2 ± 8.2*
EtOAc	0.40	7.5±2.5*	85.0 ± 2.9	50.8 ± 9.5*	100.0 ± 12.6	97.4 ± 5.7	71.0 ± 6.5*	79.4 ± 5.9*
EtOH	0.40	3.4 ± 3.5*	22.5 ± 7.5*	na	50.0 ± 11.7*	100 ± 0.0	68.6 ± 10.1*	80.7 ± 9.5
WR	100%[a]	0.0 ± 0.0*	0.0 ± 0.0*	na	0.0 ± 0.0*	0.0 ± 0.0*	na	na
	50%[b]	0.0 ± 0.0*	12.5 ± 4.8*	na	0.0 ± 0.0*	0.0 ± 0.0*	na	na
WROE	0.40	22.2 ± 14.3*	87.5 ± 4.8	122.4 ± 15.0	22.2 ± 19.1*	93.8 ± 10.4	84.9 ± 10.3	90.9 ± 10.2

[a]100% and [b]50% WR dilution

* $p<0.05$ Mann Whitney U-test (Germination and Growth).

na, not enough number of roots available.

Table 2 shows the nematicidal and insect antifeedant effects of the different *L. luisieri* extracts.

A strong nematicidal activity was found for the hydrolate (WR, pH 3.2) while the EO, EtOH and WROE extracts were not active (Table 2). Liophylization of the neutralized WR (pH 6.7) gave a residue (WRNL) that resulted in a very active solution in the presence of acid water, indicating that acidification was required for the nematicidal effect of the hydrolate. A previous report on the nematicidal effects of Labiatae essential oils against *Bursaphelenchus xylophilus* showed that an oil sample from Portuguese *L. luisieri* was not nematicidal.(Barbosa *et al.*, 2010a) In this work we also found the EO inactive. However, this is the first report on the nematicidal effects of an *L. luisieri* (acid) aqueous extract.

Moderate insect antifeedant effects were found for H and EtOAc extracts against *Myzus persicae* (62% and 71% settling inhibition) and for EtOH against *Spodoptera littoralis* (64% feeding inhibition). Similar effects have been reported for *L. luisieri* EO and EtOH extracts from this population, while supercritical CO_2 extracts exhibited increased antifeedant effects against these insects.(Julio *et al.*, 2014)

The new biocidal effects (phytotoxic, nematicidal) found for the organic extracts and the aqueous residue further supported the interest of *L. luisieri* as an added value crop plant for biopesticide development. Therefore, we proceeded with the chemical characterization of these extracts to identify specific biomarkers.

Workup of the H, PEO (vapour pressure essential oil) and WR extracts led to the isolation of seven necrodane derivatives (**1**-**7**) five of them (**1**-**3**, **5** and **7**) being described here for the first time, whereas **6** (characterized as methyl ester **6a**) and **4** were previously reported.(Baldovini *et al.*, 2005a) Additionally camphor (**9**) and 10-hydroxy-4(5)-cadinen-3-one (**10**),(Stærk *et al.*, 2004) were also identified. When the H extract was cooled to RT, an insoluble fraction was separated from the solution. The study of this insoluble material led to the isolation of the known triterpene acids: tormentic (**11**),(Numata *et al.*, 1989) and ursolic (**12**).(Seo *et al.*, 1981; Numata *et al.*, 1989) The EtOAc and EtOH extracts contained a mixture of polyphenols with rosmarinic acid (**13**),(Kuhnt *et al.*, 1994) being the major component.

Table 2. Antifeedant and Nematicidal effects of *L. luisieri* essential oil (EO), hexane (H), ethyl acetate (EtOAc), ethanolic (EtOH), hydrolate (WR) and its organic fraction (WROE) extracts.

Extract	pH	Concentration	*M. javanica*	Concentration	*S. littoralis*	*M. persicae*
		(mg/mL)	(J2 mortality %)[a]	(µg/disk)	FI[b]	SI[c]
EO		1.0	9.0 ± 1.0	100	47.0 ± 9.0	58.0 ± 8.0
H		1.0	14.2 ± 3.1	100	38.9 ± 15.1	61.8 ± 6.9*
EtOAc		1.0	10.0 ± 6.7	100	25.9 ± 14.4	71.2 ± 6.8*
EtOH		1.0	17.3 ± 2.2	100	64.0 ± 11.0	42.0 ± 10.1
WR	3.2	100% [d]	100 ± 0.0	-	-	-
		25%	48.9 ± 3.9	-	-	-
		12.5%	2.2 ± 0.6	-	-	-
WRNL	6.7	1.0	0.1 ± 0.4	-	-	-
	3.68	1.0	100 ± 0.0	-	-	-
		0.5	88.8 ± 4.0	-	-	-
		0.25	55.6 ± 2.7	-	-	-
		0.12	2.3 ± 0.7	-	-	-
WROE		1.0	7.4 ± 1.4	100	37.8 ± 16.0	nt

[a]Corrected according to Scheider-Orelli's formula.
[b]Percent feeding inhibition. [c]Percent settling inhibition. (%FI / %SI = [(1-(T/C))*100] where T = consumption /settling on treated disk; and C = consumption / settling on Control disk.
[d]WR dilutions
* p<0.05 Wilcoxon signed-rank test.
nt, not tested.

The molecular formula of **1** was determined as $C_{10}H_{16}O_2$ by HREIMS ($[M]^+$, m/z 168.1147). This formula and the NMR data (Table 3) suggested a monoterpene with three degrees of unsaturation, possessing a cyclopentane structure with a *gem*-dimethyl group and two methyl groups on double bond. The IR spectrum showed two absorption bands at ν_{max} 3406 cm^{-1} and 1686 cm^{-1} due to a hydroxyl group and a α,β-unsaturated ketone, respectively. The hydroxyl group corresponded to a primary alcohol as deduced from NMR data. The ^{13}C NMR spectrum confirmed the presence of a tetra substituted double bond conjugated with a ketone group. These data are in agreement with a necrodane skeleton and therefore, the structure of **1** was assigned as 5-(hydroxymethyl)-2,3,4,4-tetramethylcyclopent-2-enone.

Compound **2** showed a molecular formula $C_{12}H_{18}O_3$ established by HREIMS ($[M]^+$, m/z 210.1254). Its IR showed absorption bands due to an acetate group (1734 cm^{-1}) and a α,β-unsaturated ketone (1668 cm^{-1}). The NMR (Table 3) indicated the presence of a necrodane structure closely related to **1**, the main differences being the strong deshielding of the H-6 signals ($\Delta\delta = \delta$ **2** - δ **1** = 0.70 and 0.41 ppm) and the presence of signals corresponding to an acetyl group. These data allowed us to identify **2** as 2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl-methyl acetate.

Compound **3** showed a molecular formula $C_{10}H_{16}O_3$ established by HRESI-TOFMS ($[M + Na]^+$, m/z 207.0991). The NMR (Table 3) indicated the presence of a necrodane structure closely related to **1,** the main differences being the strong deshielding of the C-5 signal ($\Delta\delta = \delta$ **3** - δ **1** = 20.4 ppm) and the absence of the proton H-5. These data allowed us to identify **3** as 5-hydroxy-5-(hydroxymethyl)-2,3,4,4-tetramethylcyclopent-2-en-1-one.

The molecular formula of **5** was determined as $C_{10}H_{14}O_2$ by HREIMS ($[M]^+$, m/z 166.0991). Its IR spectrum showed absorption bands characteristic of a hydroxyl group (3427 cm^{-1}) and a carbonyl group (1684 cm^{-1}). The hydroxyl group corresponded to a primary allyl alcohol as shown in the NMR spectra (Table 3). These data together with other signals observed in the NMR spectra showed that **5** is a hydroxyl derivative of **4**. A careful study of the HMBC and NOESY correlations (Figure 1) allowed us to unambiguously determine the spatial arrangement of these substituents on the ring and therefore **5** was identified as 2-(hydroxymethyl)-3,4,4-trimethyl-5-methylenecyclopent-2-enone.

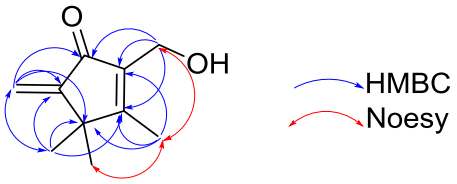


Figure 1. HMBC and NOESY correlations of compound **5**

The molecular formula of **7** was determined as $C_9H_{13}O_3$ by HREIMS ($[M+H]^+$, m/z 169.0861). This formula suggested a monoterpene with four degrees of unsaturation. Its IR spectrum showed three major absorption bands at v_{max}. 1783, 1737, 1052 due to the presence of a CO-O-CO group. The IR and NMR data (Table 3) allowed us to establish for **7** a structure of 2H-pyran-2,6(3H)-dione with a *gem*-dimethyl group and two methyl group on double bond. These data allowed us to identify **7** as 3,3,4,5-tetramethyl-2H-pyran-2,6(3H)-dione, a new nor-necrodane compound.

The necrodane carbon skeleton (1,2,2,3,4-pentamethylcyclopentane) of **1**-**7** indicates irregular unions between the two isoprene units, suggesting that their biosynthesis did not involve prenylation between the monoterpene precursors, DMAPP and IPP. Therefore, the involvement of DMAPP and isoprene (derived from the removal of HOPP from IPP) in their biosynthesis has been proposed.(Figadère *et al.*, 2007) Enzymes with acid centers (prenyltransferase and / or cyclase) could selectively protonate the di-substituted double bond of the isoprene leading to prenylation-cyclization-deprotonation that will form necrodols. These necrodols could be the precursors of other necrodanes found in *L. luisieri* through oxidation, elimination and acetylation (Scheme 1). The formation of the nor-necrodane **7** could result from the oxidation of **1** to the carboxylic acid II and the degradation of its cyclopentane ring to IV through the formation of the enol III. Then the α-ketocarboxylic decarboxylation of IV will lead to **7** (Scheme 1).

Workup of the neutralized-lyophilized residue (WRNL) resulted in the isolation of compound **8**. The molecular formula of **8** was determined as $C_{10}H_{17}O_3$ by HREIMS ($[M]^+$, m/z 185.1181) indicating two degrees of unsaturation. The 1D- and 2D-NMR data (Table 4) were concordant with a p-menthane skeleton with three hydroxyl groups at the C-1, C-2 and C-8 positions, and one double bond at the C-5 and C-6 positions. (Naik *et al.*, 1986) The observed NOE effects of H-2β with CH_3-7 and H-4 in the NOESY spectrum indicated *syn* relationship between the hydroxyisopropyl group at the C-4 position and hydroxyl groups at the C-1 and C-2 positions (Figure 2). These data allowed us to identify **8** as ($1R^*$,$2S^*$,$4R^*$)-p-menth-5-ene-1,2,8-triol.

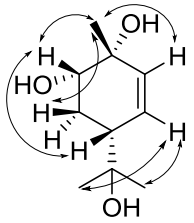
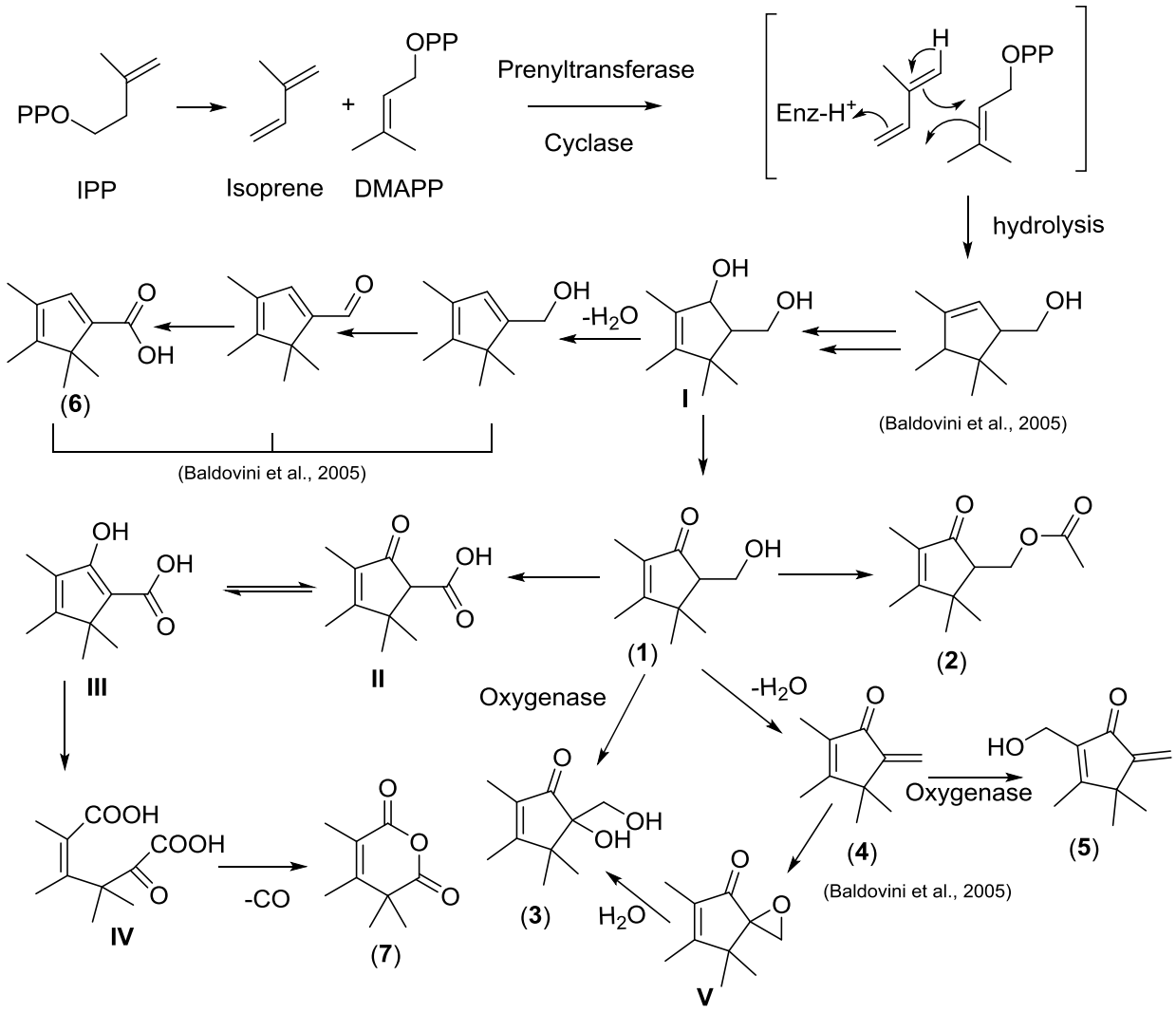


Figure 2. Significant NOESY correlations of compound **8.**

Table 5 shows the results of the phytotoxic effects of the pure compounds. Overall, *L. perenne* was more sensitive than *L. sativa*. Necrodanes **1**, **2**, **6**, **6a**, and **7**, cadinane **10** and rosmarinic acid **13** inhibited the germination of *L. sativa* between 24-72h (up to168 h for **6a**), but only **7** reduced its root growth at all the doses tested. Compounds **1**, **2**, **5**, **6** and **7** inhibited germination and growth of *L. perenne*. Cadinane **10** showed similar effects. The new nor-necrodane **7** was the only compound with phytotoxic effects against both plant species.

Compound **7** was nematicidal and **6** showed moderate effect while **3** and **8**, isolated from the active aqueous residue (WR), had no effect (Table 6). This is the first report on the nematicidal effects of this class of compounds.



Scheme 1. Possible biosynthetic pathway of necrodanes in *L. lusieri*.

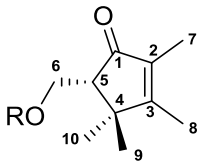
Table 3. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for compounds **1**-**3**, **5**, **6a** and **7**

	1		**2**		**3**		**5**		**6a**		**7**	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	213.6, C		205.7, C		209.2, C		196.0, C		145, CH	7.12 s	160.8, C	
2	136.4, C		134.0, C		131.3, C		137.6, C		130.3, C		119.7, C	
3	180.3, C		176.1, C		176.8, C		173.9, C		156.5, C		156.1, C	
4	46.4, C		44.4, C		47.4, C		44.4, C		53.5, C		44.5, C	
5	59.8, CH	2.30, dd (5.7, 8.9)	55.7, CH	2.47, dd (4.2, 9.2)	80.2, C		152.1, C		142.9, C		172.2, C	
6	63.2, CH_2	b 3.77, dd (5.7, 10.5) a 3.83, dd (8.9, 10.5)	62.3, CH_2	b 4.18, dd (9.2, 11.9) a 4.53, dd (4.2, 11.9)	66.2, CH_2	b 3.57, m a 3.64, m	114.4, CH_2	5.34, s 6.04, s	163.9, C		15.3, CH_3	1.97, d (0.95)
7	10.5, CH_3	1.66, s	8.3, CH_3	1.67, s	8.0, CH_3	1.72, s	56.1, CH_2	4.42, s	12.2, CH_3	1.86, d (0.95)	13.2, CH_3	1.98, d (0.95)
8	14.5, CH_3	1.94, s	11.9, CH_3	1.95, s	12.4, CH_3	1.98, s	11.3, CH_3	2.02, s	9.9, CH_3	1.79, d (0.95)	25.6, CH_3	1.49, s
9	25.0, CH_3	1.20, s	22.8, CH_3	1.23, s	19.7, CH_3	1.106, s	25.4, CH_3	1.25, s	21.4, CH_3	1.16, s	25.6, CH_3	1.49, s
10	29.2, CH_3	1.02, s	27.0, CH_3	1.09, s	24.8, CH_3	1.113, s	25.4, CH_3	1.25, s	21.4, CH_3	1.16, s		
OH		3.30, br s										
$COCH_3$			21.1, CH_3	2.05, s								
$COCH_3$			171.0, C									
OCH_3									50.7, CH_3	3.74, s		

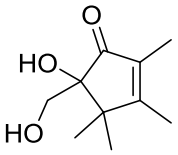
Table 4. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for compound **8**

position	δ_C, type	δ_H (J in Hz)	COSY	HMBC[a]	NOESY
1	69.8, C				
2	73.3, CH	3.86, dd (6.0, 2.8)	H-3	1, 4, 6, 8	H-7
3	27.4, CH_2	α 1.95, dd (6.0, 2.8) β 1.86, dd (13,9, 6.9)		1, 2, 4, 5	H-9, H-10 H-7, H-9, H-10
4	42.7, CH	2.35, m	H-3	3, 5, 6, 7	H-2, H-9, H-10
5	129.5, CH	5.93, dd (10.4, 2.8)	H-6, H-4	1, 3, 4	H-9, H-10
6	133.5, CH	5.75, ddd (10.4, 2.5, 0.9)	H-5	2, 4, 8	H-7
7	24.1, CH_3	1.34, s		1, 2, 6	
8	72.7, C				
9	28.3, CH_3	1.29, s		4, 7, 10	
10	26.9, CH_3	1.24, s		4, 7, 9	

[a]HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon.



1 R= H
2 R= Ac



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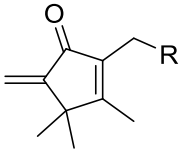
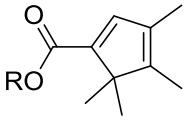
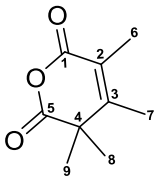


4 R= H
5 R= OH



6 R= H
6a R= Me



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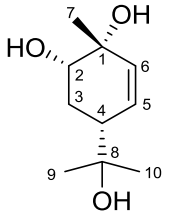

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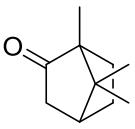

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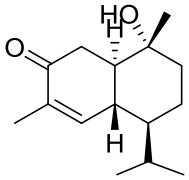

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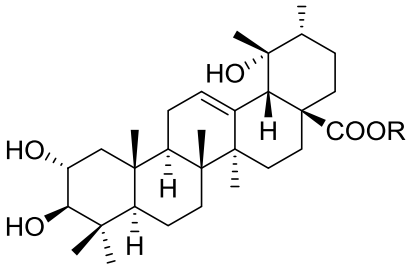
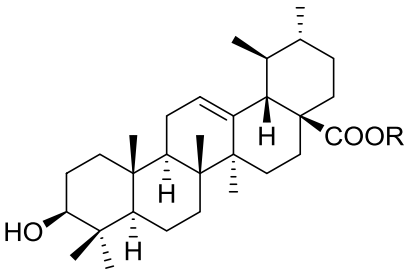
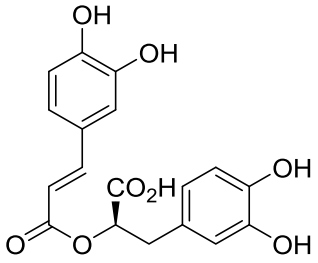


11 R = H
11a R = Me



12 R = H
12a R = Me



13

Table 5. Phytotoxic effects of compounds **1, 2, 4**-**10** and **12**,**13.**

Compound	mg/mL	*L. sativa*			*L. perenne*			
		Germination[a]		Growth[a]	Germination[a]		Growth[a]	
		24 h	144 h	Root	72 h	168 h	Root	Leaf
1	0.20	84 ± 5	100.0 ± 0.0	94.3 ± 7.2	36.7 ± 3.6*	67.6 ± 9.4	74.7 ± 9.5*	63.6 ± 9.3*
	0.10				50.0 ± 27.0*	93.5 ± 13.6	77.2 ± 13.3*	76.9 ± 16.5*
	0.05				68.8 ± 15.4*	96.8 ± 7.2	89.3 ± 11.7	94.1 ± 17.6
2	0.20	66 ± 7*	100.0 ± 0.0	88.9 ± 7.6	20.0 ± 6.7*	73.0 ± 8.3*	73.0 ± 12.6*	56.2 ± 12.7*
	0.10				36.7 ± 8.5*	59.5 ± 3.5*	88.7 ± 13.8	74.9 ± 14.0*
	0.05				75.0 ± 23.4*	83.9 ± 7.0	80.3 ± 11.3	82.5 ± 18.4
4	0.20	87 ± 9	100 ± 0.0	124.6 ± 9.6	75.0 ± 23.4*	106.5 ± 7.1	99.1 ± 12.4	104.2 ± 18.1
5	0.20	117 ± 9	108.1 ± 8.8	123.2 ± 7.9	68.8 ± 15.4*	100.0 ± 7.0	52.9 ± 7.3*	110.4 ± 14.7
6	0.20	24 ± 9*	100 ± 0.0	108.4 ± 8.2	50.0 ± 11.8*	85.3 ± 10.4	47.1 ± 6.8*	68.3 ± 10.9*
	0.10				82.7 ± 16.9	100 ± 10.2	86.5 ± 10.1	66.6 ± 10.9*
	0.05				62.5 ± 20.6*	96.8 ± 8.9	106.9 ± 12.7	112.8 ± 20.2
6a	0.20	0.0 ± 0.0*	67.5 ± 6.3*	97.8 ± 15.1	128.6 ± 11.0	100 ± 7.3	129.4 ± 18.4	123.0 ± 15.8
7	0.20	0.0 ± 0.0*	92.5 ± 2.5	61.6 ± 3.9*	33.3 ± 4.1*	83.8 ± 5.6	2.5 ± 2.8*	59.5 ± 7.5*
	0.10	5.0 ± 2.9*	95.0 ± 2.9	74.4 ± 5.2*	46.7 ± 12.9*	81.1 ± 5.8	28.9 ± 4.9*	63.6 ± 10.2*
	0.05	0.0 ± 0.0*	100.0 ± 6.1	56.9 ± 6.0*	87.5 ± 28.1	103.2 ± 8.2	86.5 ± 12.0	112.5 ± 20.5
8	0.20	87.5 ± 5.1	92.5 ± 4.8	77.1 ± 3.7*	81.3 ± 17.7	106.5 ± 10.3	99.1 ± 12.6	107.9 ± 18.1
9	0.20	100 ± 0.0	100 ± 0.0	116.3 ± 8.7	105.9 ± 16.8	92.3 ± 4.8	97.8 ± 11.9	99.5 ± 12.9
10	0.20	3.3 ± 3.3*	100 ± 0.0	151.9 ± 13.8	29.6 ± 6.1*	43.6 ± 8.8	55.2 ± 13.5*	51.6 ± 16.6*
	0.10	9.4 ± 6.0*	100.0 ± 6.1	144.8 ± 16.2	76.5 ± 15.5*	100 ± 5.8	90.6 ± 12.3	88.1 ± 14.2
12	0.20	100 ± 0.0	100 ± 0.0	107.0 ± 3.1	71.4 ± 28.0*	116.7 ± 17.3	115.0 ± 16.7	100.4 ± 13.3
13	0.20	45.5 ± 7.7*	100 ± 0.0	143.9 ± 8.2	100.0 ± 15.9	94.9 ± 3.5	93.2 ± 11.7	92.0 ± 12.9
Carvone	0.20	27.3 ± 13.5*	100 ± 0.0	95.3 ± 5.4	88.2 ± 15.0	97.4 ± 3.9	92.2 ± 11.5	100.7 ± 13.6

[a] %Control

* p<0.05 Mann Whitney U-test

Table 6. Nematicidal effects of compounds **1**-**13**.

Compound	µg/µL	J2 mortality (%)[a]	LC_{50}	LC_{90}
1	0.5	4.5 ± 1.4		
2	0.5	20.8 ± 3.4		
3	0.5	0.64 ± 0.2		
4	0.5	0.47 ± 0.3		
5	0.5	2.1 ± 0.6		
6	0.5	53.9 ± 5.1		
6a	0.5	10.1 ± 4.1		
7	0.5	88.3 ± 1.0	0.24 (0.23-0.26)[c]	0.52 (0.49-0.56)[c]
8	0.5	0 ± 0		
9	0.5	0.8 ± 1.0		
10	0.5	1.20 ± 0.3		
11a	0.5	8.3±2.8		
12a	0.5	6.7±4.2		
13	0.5	6.6±0.4		

[a] Corrected according to Scheider-Orelli's formula.

[c] Effective doses and 95% confidence limits (Probit Analysis).

Values are means of four replicates.

Necrodane-type compounds have been reported as insect antifeedants. Compound **5** showed moderate antifeedant effects against *S. littoralis* and a mixture of *trans*/*cis*-α-necrodyl acetate moderately repelled *M. persicae*.(Julio *et al.*, 2014) However, there are no reports on the phytotoxic

or nematicidal activity of necrodane-type compounds. These compounds are structurally related to cyclopentenone oxylipins, such as *cis*-(+)-12-oxo-phytodienoic acid, an inhibitor of seed germination in *Arabidopsis thaliana*.(Dave and Graham, 2012) Additionally, γ-pyrones and γ-pyridones and pyrandiones,(Asami *et al.*, 1986)ˈ(Oettmeier *et al.*, 2006) have been recognized as efficient-moderate photosystem II inhibitors. In this work the pyrandione-related compound **7** showed a strong phytotoxic effect that could be related to photosystem II inhibition. Cadinane-type sesquiterpenes showed anti-germination activity against lettuce and radish seeds,(Nawamaki and Kuroyanagi, 1996; Buchanan *et al.*, 2000) and insecticidal and ixodicidal effects.(Porter *et al.*, 1995a; Buchanan *et al.*, 2000)

The analysis of the bioactive extracts (EO, H, EtOH, WR) by GC-MS and LC-MS is shown in Table 7. Camphor (**9**) was the major component of EO and H, followed by necrodane **4**. The H extract also contained compound **1**, higher amounts of **2** than the EO and **6**. This EO showed qualitative and quantitative differences in composition respect to the wild parent population which contained higher relative amounts of camphor **9** (74%), **4** (5.3%) and *trans*-α-necrodyl acetate (8.2%).(Julio *et al.*, 2014) A similar oil composition has also been described for wild and short-term experimentally cultivated *L. luisieri*.(González-Coloma *et al.*, 2011a) The EtOH extract contained tormentic (**11**) and ursolic (**12**) acids, cadinane (**10**), necrodanes **1, 2**, **5** and rosmarinic acid (**13**). The EtOH extract from the wild parent population also showed **11** as the major component, followed by **10**.(Julio *et al.*, 2014) The WR extract contained **8**, **1**, **5**, **3** and **2** while the WRNL was enriched in **8**, **3** and **10** with respect to WR. Compounds **6** and **7** (isolated from the H and PEO extracts) could not be detected by either GC-MS or HPLC-MS and therefore we cannot rule out their presence in the WR extracts.

None of the active compounds alone explained the phytotoxic or nematicidal activity of the extracts, therefore synergistic effects for the components of the EO, EtOH, and WR extracts are suggested. For example camphor (**9**) has been shown to inhibit the germination and root elongation of *L. sativa*, *L. perenne*,(Kennedy *et al.*, 2011) (at higher doses than the ones tested here) and *Brassica campestris*, inhibiting its mitotic index and DNA synthesis in the root apical meristem,(Nishida *et al.*, 2005) and also affected membrane lipids in maize seedlings.(Zunino and Zygadlo, 2005) Olenanane type triterpenes (β-amyrin and oleanolic acid) found in *L. luisieri* showed moderate postingestive effects on *S. littoralis* larvae and moderate phytotoxicity to *L. sativa*.(González-Coloma *et al.*, 2011b) However, in this work we isolated tormentic and ursolic acids (both inactive), while β-amyrin and oleanolic acid were detected as mixtures in the insoluble hexane fraction (data not shown). Rosmarinic acid (**13**) is found in many Lamiaceae herbs, and it is supposed to act as a preformed constitutively accumulated defense compound.(Petersen and Simmonds, 2003; Petersen, 2013) Similarly, the insecticidal effects of *L. luisieri* SC extracts have been attributed to the synergistic effects of their components.(Julio *et al.*, 2014)

Table 7. Chemical composition of the bioactive *L. luisieri* extracts

Compound	EO	H	EtOH	WR	WRNL
	GCMS		LCMS		
Camphene	1.57				
1.8-cineole	1.95	0.53			
Fenchone	2.85	1.26			
Camphor (**9**)	60.33	55.49			
2.3.4.4-tetramethyl-5-methylidenecyclopent-2-en-1-one (**4**)	8.48	11.29			
D-Verbenone	1.17	0.92			
Trans-α-Necrodyl acetate		2.24			
Exobornyl acetate	4.64				
Cis-α-Necrodyl acetate	1.90				
5-(2-hydroxypropan-2-yl)-2-methylcyclohex-3-ene-1.2-diol (**8**)				2.60	20.33
5-Hydroxymethyl-2.3.4.4-tetramethylcyclopent-2-en-1-one (**1**)		3.58	6.65	20.99	
2-(hydroxymethyl)-3.4.4-trimethyl-5-methylenecyclopent-2-en-1-one (**5**)	tr	0.7	2.9	2.55	
5-hydroxy-5-(hydroxymethyl)-2,3,4,4-tetramethylcyclopent-2-en-1-one (**3**)		0.7		3.48	12.95
Rosmarinic acid (**13**)			2.56		
(2.2.3.4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate (**2**)	0.66	7.18	7.88	26.70	
(1R.6R.7S.10R)-10-Hydroxy-4(5)-cadinen-3-one (**10**)			8.95		4.42
Tormentic acid (**11**)			11.34		
Tormentic acid isomer			1.72		
Oleanolic acid			2.57		
Ursolic acid (**12**)			9.72		

In conclusion, the hydrolate (WR / WRNL) was the most phytotoxic and nematicidal extract of experimentally cultivated *Lavandula luisieri*, followed by the hexanic (H), ethanolic (EtOH) and essential oil (EO). The chemical study of the H, EO and WR extracts led to the isolation of new (**1**-**3**, **5** and **7**) and known (**4** and **6**) necrodanes, one cadinane sesquiterpene (**10**), camphor (**9**), ursolic (**11**) and tormentic acids (**12**). Rosmarinic acid (**13**) was isolated from the EtOAc extract and the new compounds **3** and **8** from the hydrolate WR. Compounds **1**, **4**-**7**, **6a** and **10** showed phytotoxic effects with varying potencies. Only compound **7** was nematicidal, therefore this nor-necrodane could be a lead compound for the synthesis of new herbicidal and nematicidal agents. The analysis of the bioactive extracts showed that camphor (**9**) was the major component of EO and H, followed by necrodane **4**. The H extract also contained compound **1**, higher amounts of **2** than the EO and **6**. The EtOH extract contained tormentic (**11**) and ursolic (**12**) acids, cadinane (**10**), necrodanes **1, 2**, **5** and rosmarinic acid (**13**). The WR extract contained **8**, **1**, **5**, **3** and **2** while the WRNL was enriched in **8**, **3** and **10.** Synergistic phytotoxic and nematicidal effects for the components of these extracts are suggested.

EXPERIMENTAL

General. NMR spectra (1H and 13C) were measured on a Bruker AMX2 500 MHz and Varian Direct-Drive 500 (1H 500 MHz/13C 125 MHz) spectrometers with pulsed field gradient using the solvent as internal standard (CDCl3, at δH7.26 and δC77.0). The programs used in two-dimensional (2D) NMR experiments (HMBC, HSQC, COSY, and NOESY) were those furnished with the manufacturer's software. For high-resolution MS we used an Autospec-Q VG-Analytical (Fisons) mass spectrometer and Micromass Autospec instrument at 70 eV. For IR spectra we used a Mattson Model Satelite FTIR spectrometer.

Plant material and cultivation.

L. luisieri plants have been cultivated in an experimental field located in Comarca del Campo de Cariñena, Aguarón (Zaragoza, Spain) (16 m, 41º 19' 13.33'' N; 1º 19'53.9'' W). The experimental design consisted of three random blocks (2 m between blocks), containing four 10 m rows with 104 plants per row (49.92 m2) at a distance of 1.20 x 0.40 m (0,48 m2/plant). The experimental field was established in march 2008 with plants produced from seeds collected in June 2007 from a wild population located in Pueblo Nuevo del Bullaque (Ciudad Real, Spain; latitude: 39° 27' 41'' N, longitude: 4° 24' 34'' W, altitude: 733 m) and germinated in a commercial nursery. Flowering aerial parts of the wild and cultivated plants collected in June 2009 were dried in the shade at room temperature and ground for extraction.

Extraction and fractionation.

Hydrodistillation (EO) was performed in a Clevenger-type apparatus (0.8% yield) according to the method recommande by European Pharmacopoeia (http://www.edqm.eu/en/Homepage-628.html). Pilot plant vapor pressure extraction (PEO, 0.2 % yield) was carried out in a stainless steel distillation plant equipped with a 100 Kg distillation chamber, a 500 L vessel and a pressure range of 0.5-1.0 bar. The water collected after the essential oil was decanted (1.16 L) was filtered to give an acidic water residue (WR, 4.5 mg/mL of organic extract, pH 3.2). 155 mL of WR were extracted with dichloromethane (DCM, 150 mL x 3) to give an organic fraction (WROE) (230 mg, 0.15 % yield). 50 mL of WR were neutralized at pH 6.62 with NaOH 2N and lyophilized to give a dry residue (WRNL, 36.7 mg, 0.073% yield). The organic extractions (hexane, H; ethyl acetate, EtOAc and ethanol, EtOH) were carried out in a Soxhlet for 12 h (131g, 1.2%; 0.76 % and 12.5 % yield respectively). The insoluble material from the H extraction (1.60 g) was filtered, and the solution obtained was washed with 2N NaOH solution. The aqueous layer was acidified with 2N HCl at pH 2 and extracted with t-butylmethylether (E). Both organic layers were washed with brine, dried with anhydrous Na2SO4, filtered and concentrated in vacuo to afford 687 mg and 724 mg of neutral and acid fractions respectively.

The soluble H extract (8.4 g) was fractionated by column chromatography over silica gel (Si-gel) using H:E mixtures of increasing polarity, affording camphor (9) (714 mg),(Demarco et al., 1969; Barrero et al., 2005) 4 (103 mg), 2 (148 mg), 1 (86 mg) and 10 (360 mg).(Stærk et al., 2004) Additional workup of the H extract (3.1g) by flash Si-gel column chromatography eluted with a H:EtOAc gradient (0-30 % EtOAc) at 50 mL/min gave 8 fractions. Compound 10 (110 mg) was isolated from fraction 7. Further chromatography of fraction 5 on a 20 g Si pre-packed flash cartridge (ExtraBond Flash OT SI

20g 70 mL 26.8 x 154 mm Scharlau) eluted with H:EtOAc gave compound 7 (5 mg). Fraction 8 was chromatographed similarly and eluted with H:EtOAc to yield compound 5 (9 mg).

The acid insoluble material (724 mg) was fractionated by column chromatography over Si-gel using H:E:EtOAc mixtures of increasing polarity to yield six fractions. Fraction 6 (EtOAc, 154 mg) was methylated with TMSCHN2,(Hasimoto et al., 1981) to afford a crude product that was subjected to column chromatography over Si-gel to yield methyl ursolate (12a) (30 mg) and methyl tormentate (11a) (79 mg).(Seo et al., 1981; Numata et al., 1989)

The plant material extracted with H was further extracted with EtOAc. This EtOAc extract (1g) was fractionated by column chromatography over Si-gel using H:E mixtures of increasing polarity to yield rosmarinic acid (13) (30 mg).(Kuhnt et al., 1994)

Essential oil (PEO, 52 g) was added to a Na2CO3 solution (25 mg/mL) and stirred for 2 hours. The water layer was treated with 10 g of NaCl, extracted with dichloromethane (3 x 200 mL) and acidified with diluted HCl to pH 3. The acid extract was partitioned with dichloromethane (3 x 200 mL), the resulting organic extract dried over Na2SO4 and the solvent evaporated to give 480 mg of a crystalline yellow solid which was purified by flash chromatography to give 200 mg of compound 6 (white solid) and 20 mg of 7.

The organic fraction WROE (230 mg) was fractionated by column chromatography over silica gel (Si-gel) using H: EtOAc mixtures of increasing polarity, affording compound 3 (3.5 mg). Neutralized-lyophilized hydrolate WRNL (110 mg) was chromatographed on a Si-gel column eluted with EtOAc to yield compound 8 (3 mg).

Compound 1. Colorless syrup; $[\alpha]_D$ -9.7 (c1, CH_2Cl_2); IR (film) ν_{max} 3406, 2960, 2931, 2875, 1686, 1465, 1378, 1330, 1240, 1041 cm^{-1}; ^1H NMR data ($CDCl_3$, 500 MHz) see Table 3; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3; HREIMS m/z 168.1147 [M]+ (calcd for $C_{10}H_{16}O_2$, 168.1150); EIMS 70 eV m/z (rel. int.): 168 $[M]^+$ (53), 138 (100), 135 (80), 123 (99), 109 (44), 107 (79), 81 (49), 79 (39), 67 (32), 41 (34).

Compound 2. Colorless syrup; $[\alpha]_D$ -7.9 (c1, CH_2Cl_2); IR (film) ν_{max} 2953, 2928, 2859, 1734, 1668, 1452, 1370, 1262, 1231, 1093, 1017 cm^{-1}; ^1H NMR data ($CDCl_3$, 500 MHz) see Table 3; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3; HREIMS m/z 210.1254 $[M]^+$ (calcd for $C_{12}H_{18}O_3$, 210.1256); EIMS 70 eV m/z (rel. int.): 210 $[M]^+$ (8), 151 (19), 150 (33), 136 (11), 135 (100), 123 (14), 107 (41), 91 (10), 79 (9), 43 (33), 41 (10).

Compound 3. Colorless syrup; $[\alpha]_D$ -7.0 (c0.24, $CHCl_3$); ^1H NMR data ($CDCl_3$, 500 MHz) see Table 3; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3; HRESI-TOFMS m/z 207.0991 $[M + Na]^+$ (calcd for $C_{10}H_{16}O_3$, 207.0997); EIMS 70 eV m/z (rel. int.): 184 $[M]^+$ (1), 154 (100), 151 (29), 136 (53), 125 (47), 123 (40), 121 (29), 107 (23), 81 (20), 55 (24), 43 (46).

Compound 5. ^1H NMR data ($CDCl_3$, 500 MHz) see Table 3; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3; HREIMS m/z 166.0991 $[M]^+$ (calcd for $C_{10}H_{14}O_2$, 166.0994); EIMS 70 eV m/z (rel. int.): 166 $[M]^+$ (76), 151 (100), 149 (21), 137 (30), 135 (29), 123 (54), 105 (26), 95 (35), 91 (27), 79 (25), 67 (37), 59 (16).

Compound 6a. ^{1}H NMR ($CDCl_3$, 500 MHz) see Table 3; ^{13}C NMR ($CDCl_3$, 125 MHz) see Table3; HREIMS m/z 180.1144 $[M]^+$ (calcd for $C_{11}H_{16}O_2$, 180.1150). EIMS 70 eV m/z (rel.int.): 180 $[M]^+$ (13), 165 (47), 157 (33), 137 (39), 125 (59), 107 (38), 99 (61), 91 (74), 71 (82).

Compound 7. IR (film) ν_{max} 2925, 1783, 1737, 1052 cm^{-1}; ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 3; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 3; HREIMS m/z 169.0861 $[M + H]^+$ (calcd for $C_9H_{13}O_3$, 169.0865). EIMS 70 eV m/z (rel. int.): 169 $[M+H]^+$ (11), 124 (66), 123 (20), 109 (60), 96 (9), 81 (100), 79 (12), 53 (12).

Compound 8. Colorless syrup; $[\alpha]_D$ -15.7 (c0.28, $CHCl_3$); ^{1}H NMR data ($CDCl_3$, 500 MHz) see Table 4; ^{13}C NMR data ($CDCl_3$, 125 MHz) see Table 4; HREIMS m/z 185.1181 $[M-H]^+$ (calcd for $C_{10}H_{17}O_3$, 185.1178); EIMS 70 eV m/z (rel.int.): 185 $[M-H]^+$ (1), 168 (2), 153 (4), 135 (4), 110 (94), 109 (55), 107 (11), 95 (77), 91 (12), 81 (12), 67 (20), 59 (100).

GC-MS Analysis

EO and H extracts were analyzed by GC-MS using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, California, USA) coupled to an Agilent 5973N mass detector (electron ionization, 70 eV) (Agilent Technologies, Palo Alto, California, USA) and equipped with a 25m x 0.2 mm id HP-1 (methyl polisiloxane, 0.2 µm film thickness) and a 30m x 0.25mm id Carbowax (polyethylene glycol, 0.25 µm film thickness) capillary columns (Hewlett-Packard). Working conditions were as follows: injector temperature, 260 ºC; temperature of the transfer line connected to the mass spectrometer, 280 ºC; column temperature 70 -190ºC, 5 ºC min^{-1}. EI mass spectra and retention data were used to identify compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001) and pure samples (**1**-**5**, **8**, **10**). Compounds **6** and **7** were also injected but not detected. Quantitative data were obtained from the TIC peak areas without the use of response factors.

HPLC-MS Analysis

The EtOH extract was analyzed by HPLC-MS on a Shimadzu LC-20AD HPLC coupled to a LCMS-2020 QP mass spectrometer using an electrospray ionization (ESI) interface and a Teknokroma, Mediterranea Sea_{18} column (250 x 4.6 mm, 5 µm particle size) with an ACE 3 C18 analytical guard cartridge. The compounds were eluted with methanol (MeOH): 0.1% acetic acid in milli-Q water 38:100% gradient for 45 min, 100% MeOH for 10 min and 100:38% for 13 min at 0.5 mL/min and 15 L/min nitrogen (drying gas for solvent evaporation) flow rates. The electrospray capillary potential was set to +4.50kV and ESI was conducted in the Full Scan positive mode (m/z = 145-545) with a potential of 1.30 kV and a capillary temperature of 250º C. Stock solutions of extracts (0.25 µg/µL), compounds **1**-**5**, **8**, **10**, **12, 13** and oleanolic acid (Sigma) (0.05 µg/µL) were dissolved in MeOH for sample injection (10 µL). Compounds **6** and **7** were also injected but not detected. All the solvents used were HPLC-MS grade.

Insect Bioassays

S. littoralis and *M. persicae* colonies were reared on artificial diet and bell pepper (*Capsicum annuum*) plants respectively, and maintained at 22 ± 1ºC, >70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber. Bioassays were conducted with newly emerged *S. littoralis* L6 larvae or ten *M. persicae* adults as described by Burgueño-Tapia et al., (2008). The organic extracts and pure compounds were tested at initial concentrations of 100 and 50 μg/cm^2 respectively.

Phytotoxic activity

The experiments were conducted with *Lactuca sativa cv* Teresa (Fito, España) and *L. perenne* seeds (100 seeds / test) in 12-well microplates as described.(Martín *et al.*, 2011) The organic extracts and pure compounds were tested at initial concentrations of 0.4 and 0.2 mg/ml (final concentration in the well) and diluted serially if needed. The hydrolate was tested without dilution (100%) and then was diluted serially. Germination was monitored for 6 (*L. sativa*) or 7 days (*L. perenne*) and the root length measured at the end of the experiment (25 plants randomly selected for each experiment, digitalized and measured with the application Image J, http//rsb.info.nih.gov./ij/). A no parametric analysis of variance (ANOVA) was performed on radical length data. Carvone (5μg/μL) was included as a positive control.(De Martino *et al.*, 2010)

Nematode bioassays

A *Meloydogine javanica* population maintained on *Lycopersicon esculentum* plants (var. Marmande) in pot cultures at 25±1 °C, >70% relative humidity has been used. Second stage juveniles (J2) hatched within a 24 h period from egg masses handpicked from infected tomato roots were used. The experiments were carried out in 96-well microplates (Becton, Dickinson) as described.(Andres *et al.*, 2012) The organic extracts and pure compounds were tested at initial concentrations of 1.0 and 0.5 mg/ml (final concentration in the well) and diluted serially if needed. The hydrolate was diluted serially. The number of dead J2 was recorded after 72 hours. All treatments were replicated four times. The data is presented as percent mortality corrected according to Scheider-Orelli's formula. Effective lethal doses (LC_{50} ans LC_{90}) were calculated for the active pure compounds by Probit Analysis (5 serial dilutions).

ACKNOWLEDGEMENTS

This work has been partially supported by grants CTQ2012-38219-C03-01 (Spain), Junta de Andalucía (Excellence Project P08-FQM-3596) Spain and JAE-CSIC (predoctoral fellowship to L.F. Julio).

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4.8. Ixodicidal compounds from cultivated *Lavandula luisieri*

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Manuscript in preparation **c**

Abstract

Several extracts from cultivated Lavandula luisieri (essential oil, EO; hexane, Hx and the organic fraction of the residual hydrolate, OE0) showed increasing larvicidal effects against the hard tick Hyalomma lusitanicum. The presence of necrodane-type compounds in these extracts explained these effects, being 3,3,4,5-tetramethyl-2H-pyran-2,6(3H)-dione (6) and (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate (2) and the most potent ones with activity levels within the range of the positive control thymol. These compounds represent a new class of ixodicidal agents.

Key words: Lavandula luisieri, cultivated, essential oil, hexane, hydrolate, Hyalomma lusitanicum, necrodane-type, larvicidal.

1. Introduction

Hard ticks (Ixodidae), are a broad group of hematophagous with 720 species that colonize a wide range of cold- and hot-blooded vertebrate hosts. Ticks can transmit a great variety of pathogens including helminths, protozoa, rickettsiae, spirochetes, other bacteria and viruses to vertebrates, being the first vectors affecting wild and domestic animals and livestock and the second most important vectors affecting humans after mosquitoes (Guglielmone *et al.*, 2014).

The genus *Hyalomma* is a relatively young phylogenetic group of ixodid ticks, well adapted to arid biotopes of the Old World. The domestication and development of cattle-breeding stimulated the evolution and biological progress of this group (Kolonin, 2009). *Hyalomma lusitanicum*, Koch 1844 (Ixodoidea: Ixodida) is abundant in the Mediterranean region (Apanaskevich *et al.*, 2008), has a widespread distribution some regions of Southern Spain (Encinas-Grandes, 1986), and it is becoming much more common in central Spain (Basco-Basco *et al.*, 2008), (Barandika *et al.*, 2011). *H.lusitanicum* is one of the vectors of *Theileria annulata* that causes Mediterranean theileriosis in cattle and it is a potential vector of several zoonotic bacterial agents such as *Anaplasma* spp., *Ehrlichia* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *Francisella* spp. and *Rickettsia* spp. (Toledo *et al.*, 2008), (Toledo *et al.*, 2009) including Crimean-Congo Hemorrhagic fever virus (CCHFV) (Estrada-Peña *et al.*, 2012).

Currently, tick control relies mostly on rapid-acting chemical pesticides (Ostfeld *et al.*, 2006). However, the use of synthetic acaricides has increased the selection of acaricide-resistant ticks, environmental contamination and drug residues (Kiss *et al.*, 2012). Plant extracts, including essential oils and their terpene components have been reported as being toxic and/or repellent to ticks (Kiss *et al.*, 2012), (Cruz *et al.*, 2013), (Kröber *et al.*, 2013) and therefore are a promising new source of natural and safer ixodicidal agents.

Lavandula luisieri (Rozeira) Riv.-Mart. (Rivas-Martínez, 1979), is a small aromatic shrub endemic to the Iberian Peninsula. Previous studies showed that *L. luisieri* oil contained 1,8-cineole, lavandulol, linalool and their acetates, also present in other *Lavandula* species, in addition to a series of compounds with a 1,2,2,3,4-pentamethylcyclopentane (necrodane) structure (García-Vallejo *et al.*, 1994), (Lavoine-Hanneguelle and Casabianca, 2004), (Baldovini *et al.*, 2005b), (Julio *et al.*, submitted for publication). *L. luisieri* essential oil showed antifungal and antibacterial effects (Lavoine-Hanneguelle and Casabianca, 2004), (Zuzarte *et al.*, 2012).

The major components of the *L. luisieri* essential oils from central and southern populations of the Iberian Peninsula were camphor, 1,8-cineole and 2,3,4,4-tetramethyl-5-methylen-2-cyclopenten-1-one (Sanz *et al.*, 2004). These oils showed moderate insect antifeedant effects (González-Coloma *et al.*, 2006). The essential oils of western *L. luisieri* populations had trans-α-necrodyl acetate as the major component, showed less variability in their composition and had stronger insect antifeedant effects (González-Coloma *et al.*, 2011a). The supercritical extraction (SCE) of *L. luisieri* improved the concentration of necrodane-type ketones and exhibited stronger insect antifeedant effects than the essential oil and ethanolic extract (Julio *et al.*, 2014). Furthermore the EO, hydrolate (WR) and hexane (Hx) extract from a pre-domesticated population showed phytotoxic (EO, Hx) and nematicidal (WR) effects, explained by their content in necrodane-type compounds (Julio *et al.*, submitted for publication).

In this work we have studied the ixodicidal effects on *H. lusitanicumm* larvae of different extracts from *L. luisieri* plants under domestication (essential oil, EO; hexane and ethanolic extract, Hx, EtOH and the hydrolate organic fraction, OE0) and their chemical composition. The compounds isolated from the active extracts have been also tested.

2. Materials and Methods

2.1. Plant material and cultivation

L. luisieri plants have been cultivated in an experimental field located in Comarca del Campo de Cariñena, Aguarón (Zaragoza, Spain) (16 m, 41º 19' 13.33'' N; 1º 19'53.9'' W) as described in Julio et al. (Julio et al., submitted for publication). The experimental design consisted of three random blocks (2 m between blocks), containing four 10 m rows with 104 plants per row (49.92 m2) at a distance of 1.20 x 0.40 m (0,48 m2/plant). The experimental field was established in march 2008 with plants produced from seeds collected in June 2007 from a wild population located in Pueblo Nuevo del Bullaque (Ciudad Real, Spain; latitude: 39° 27' 41'' N, longitude: 4° 24' 34'' W, altitude: 733 m) and germinated in a commercial nursery. The aerial parts of the cultivated plants collected during 2009-2012, were dried in the absence of light at room temperature.

2.2. Extraction

The hexanic (Hx) and ethanolic extractions (EtOH) were performed in a Soxhlet apparatus with n-hexane or EtOH and concentrated in vacuo (1.2 and 12.5 % yield respectively).

Laboratory scale hydrodistillation (essential oil, EO) was performed in a Clevenger-type apparatus (0.8% yield) according to the method recommended by European Pharmacopoeia (http://www.edqm.eu/en/Homepage-628.html). Pilot plant vapor pressure extraction (PEO, 0.2 % yield) was carried out in a stainless steel distillation plant equipped with a 100 Kg distillation chamber, a 500 L vessel and a pressure range of 0.5-1.0 bar. The water collected after the essential oil was decanted (1.16 L) was filtered to give an acidic EO-free water residue (WR, 4.5 mg/mL of organic components, pH 3.2).
The water collected after the essential oil was decanted and filtered to give an EO-free water residue (WR0, 4.5 mg/ml of organic components, pH 3.2). WR0 (1000 ml) was extracted with dichloromethane (DCM, 800 ml x 3) to give the organic extract OE0 (3.42 g, 0.34 % yield).

2.3. Organic extract OE0 fractionation

The organic extract OE0 was chromatographed by flash chromatography on a 2.5 cm diameter silica cartridge (40-70 µm) eluted with a DCM:MeOH gradient (100:0-95:5, 20 ml/min) (Jones Flash Chromatography). The fractions were monitored by TLC (silica gel 60 F254, 0.25 mm, Merck) to give fractions OE1 (1.1%), OE2 (12.0%), OE3 (4.3%) and OE4 (6.6%).

2.4. CG-MS analysis

The essential oil (EO), hexane (Hx), hydrolate organic extract (OE0) and fractions (OE1-4) were analyzed by GC-MS using an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, California, USA) coupled to an Agilent 5973N mass detector (electron ionization, 70 eV) (Agilent Technologies, Palo Alto, California, USA) and equipped with a 25m x 0.2 mm id HP-1 (methyl polisiloxane, 0.2 µm film thickness) and a 30m x 0.25mm id Carbowax (polyethylene glycol, 0.25 µm film thickness) capillary columns (Hewlett-Packard). Working conditions were as follows: injector temperature, 260 ºC; temperature of the transfer line connected to the mass spectrometer, 280 ºC; column temperature 70-190ºC, 5 ºC min-1. EI mass spectra and retention data were used to identify compounds by comparing them with those of standards or found in the Wiley Mass Spectral Database (2001). Quantitative data were obtained from the TIC peak areas without the use of response factors.

2.5. HPLC-MS analysis

The EtOH extract was analyzed by HPLC-MS on a Shimadzu LC-20AD HPLC coupled to a LCMS-2020 QP mass spectrometer using an electrospray ionization (ESI) interface and a Teknokroma, Mediterranea Sea_{18} column (250 x 4.6 mm, 5 µm particle size) with an ACE 3 C18 analytical guard cartridge. The compounds were eluted with methanol (MeOH): 0.1% acetic acid in milli-Q water 38:100% gradient for 45 min, 100% MeOH for 10 min and 100:38% for 13 min at 0.5 mL/min and 15 L/min nitrogen (drying gas for solvent evaporation) flow rates. The electrospray capillary potential was set to +4.50kV and ESI was conducted in the Full Scan positive mode (m/z = 145-545) with a potential of 1.30 kV and a capillary temperature of 250º C. Stock solutions of extracts (0.25 µg/µL).
Compounds **1**, **2**, **4**, **6**, **7**, **9**, **11** and **12** (isolated from *L. luisieri* EO and Hx extracts (Julio *et al.*, submitted for publication)) and oleanolic acid (Sigma) (0.05 µg/µL) were dissolved in MeOH for sample injection (10 µL). All the solvents used were HPLC-MS grade.

2.6. Ixodicidal bioassay

Hyalomma lusitanicum Engorged females were collected in central Spain (Finca La Garganta, Ciudad Real) from their host (deer) and maintained at 22-24ºC and 70% RH until oviposition. The eggs were kept under the same environmental conditions until they hatched. Larvae (4-6 weeks old) were used for the bioassays as described in González-Coloma et al. (Gonzalez-Coloma, 2013). Briefly, 50 µl of the extract / compound solution were added to 25 mg of cellulose at different concentrations and the solvent was evaporated. The initial stock solutions were of 20 and 10 µg/µl for extract of pure compound respectively. Acibelte® (cipermetrin 0.5%) was used as synthetic pesticide control (10 µg/µl) along with a blank test (cellulose) and a negative control (solvent plus cellulose). Thymol (Sigma) was used as natural positive control for comparison (De Oliveira Monteiro *et al.*, 2010), (Cruz *et al.*, 2013). For each test, three replicates with 20 larvae each were used. Paralyzed (movement in one leg after stimulation) and dead ticks were counted after 24h of incubation with the treated cellulose at the environmental conditions described, using a binocular magnifying glass.

The larvicidal activity data are presented as percent mortality corrected according to Schneider-Orelli's formula. Effective lethal doses (LC_{50} and LC_{90}) were calculated by Probit Analysis (STATGRAPHICS Centurion XVI, version 16.1.02).

3. Results

Table 1 show the ixodicidal effects of the essential oil (EO), organic extracts (Hx, EtOH), the organic fraction of the hydrolate (OE0) and its fractions (OE0 2-4). Both the EO and the OE0 extracts were very active against the larvae with the OE0 extract being the most potent. The bioassay of the OE0 fractions indicated that the active components against *H. lusitanicum* were concentrated in fraction OE02.

Several essential oils have been reported as being toxic and/or repellent to ticks (Kiss *et al.*, 2012), (Cruz *et al.*, 2013), (Kröber *et al.*, 2013), including *Hyalomma* spp. *Tagetes minuta* EO was repellent and delayed moulting of *H. rufipes* adults (Nchu *et al.*, 2012), *Lippia javanica* EO was repellent to *H. marginatum* adults (Magano *et al.*, 2011). *Lavandula angustifolia* EO, rich in linalool and linalyl acetate, was repellent to *H. marginatum* adults (Mkolo and Magano, 2007). However, this is the first report on the acaricidal effects of *L. luisieri* EO and extracts on *H. lusitanicum* larvae.

Table 1. Toxicity of essential oil (EO), organic extracts (EtOH, Hx, OE0) and fractions (OE2, OE3, OE4) of *L. luisieri* against *H. lusitanicum* larvae.

Extract	Concentration (µg/mg cellulose)	*H. lusitanicum* larvae
EO	40	100 ± 0
	20	8.8 ± 0.6
Hx	40	78.9 ± 9.5
	20	62.0 ± 8.7
	10	25.4 ± 3.0
OE0	40	100 ± 0.0
	20	100 ± 0.0
	10	100 ± 0.0
	5	38.8 ± 1.1
OE2	40	100 ± 0.0
	20	100 ± 0.0
	10	94.2 ± 0.1
	5	71.7 ± 0.3
OE3	40	66.3 ± 6.2
OE4	40	9.9 ± 1.1
EtOH	40	10.5 ± 2.4

Table 2 shows the composition of the extracts. Camphor (**8**) was the major component of the EO, followed by 5-methylen-2,3,4,4-tetramethylcyclopenten-2-one (**3**). The EO showed qualitative and quantitative differences in composition respect to the wild parent population EO, which contained higher relative amounts of **8** (74%), **3** (5.3%) and trans-α-necrodyl acetate (8.2%) (Julio *et al.*, 2014). A similar oil composition has also been described from a wild population rich in camphor from Toledo (group 5 in Gonzalez-Coloma et al. (González-Coloma *et al.*, 2006)) and from a western Iberian population experimentally cultivated for two years (González-Coloma *et al.*, 2011a). The hexane extract (Hx) and organic extract from the

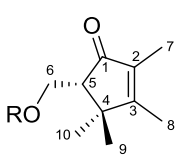
hydrolate (OE0) presented higher concentrations of **1**, **2**, **3** and **9** (only in Hx) respect to the EO. The most active fraction, OE02, contained **1** and **2** as major compounds. The EtOH extract had tormentic (**10**) and ursolic (**11**) acids, **1**, **2** and **9**. Similarly, an EtOH extract of the wild parent population also showed **10** as the major component, followed by the cadinene derivative **9** (Julio *et al.*, 2014).

Table 3 shows the activity of the compounds isolated from *L luisieri* (Julio *et al.*, submitted for publication). All the necrodane-type compounds (**1**-**6**) were ixodicidal with varying potencies, being compounds **2** and **6** the most active ones, while **1**, **3**, **4** and **5** were moderately larvicidal. It is interesting to note that the LD_{90} value of **2** and **6** had activity levels within the range of the positive control thymol and lower volatility (M+ 210 and 168 vs. 150 for thymol). The acetylation in **2** and the presence of two ketone groups in the main skeleton (**6**) resulted in strong acaricidal effects. Compund **9** was not significantly active against *H. lusitanicum* larvae, however the related cadina-4,10(15)-dien-3-one disrupted the oviposition and hatching of *Rhipicephalus (B.) microplus* (Porter *et al.*, 1995b).

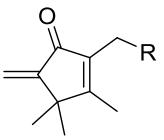
Table 2. Chemical composition of *L. luisieri* extracts

Identified compounds	EO	Hx	OE0	OE2	EtOH
	Relative %				
	GCMS				LCMS
Camphene	1.6				
1,8-cineole	2.0	0.5			
Fenchone	2.9	1.3			
Camphor (**8**)	60.3	55.5	49.4		
2,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one (**3**)	8.5	11.3	19.7		
D-Verbenone	1.2	0.9	2.5		
Trans-α-Necrodyl acetate		2.2			
Exobornyl acetate	4.6				
Cis-α-Necrodyl acetate	1.9				
5-Hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one (**1**)	tr	3.6	10.1	20.1	5.0
2-(hydroxymethyl)-3,4,4-trimethyl-5-methylenecyclopent-2-en-1-one (**4**)	tr	0.7	1.2	3.8	1.0
$(C_{10}H_{16}O_3)$[a]	tr	0.7	0.7	2.1	tr
Rosmarinic acid (**12**)					2.6
(2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate (**2**)	0.7	7.2	7.7	29.1	9.7
(1R,6R,7S,10R)-10-Hydroxy-4(5)-cadinen-3-one (**9**)	tr	3.5	tr		16.9
Tormentic acid (**10**)					15.0
Tormentic acid isomer					3.2
Oleanolic acid					2.6
Ursolic acid (**11**)					4.5

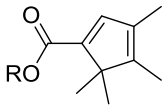
[a]Possible di-alcohol derivative of **4** (or mono-alcohol derivative of **1** or **12**)
tr, trace amounts.



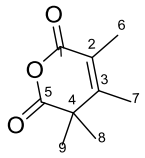
1 R= H
2 R= Ac



3 R= H
4 R= OH



5 R= H
5a R= Me



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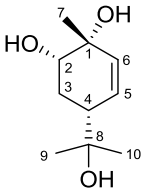

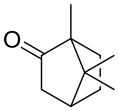

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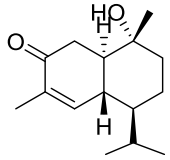

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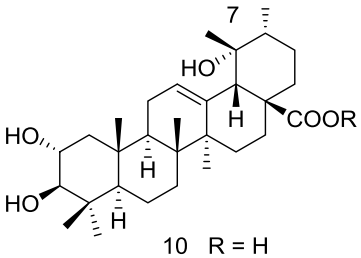
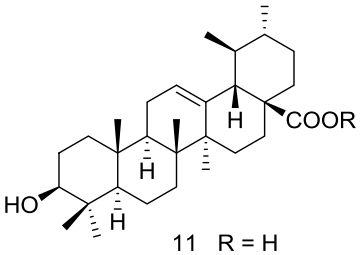


10 R = H
10a R = Me



11 R = H
11a R = Me

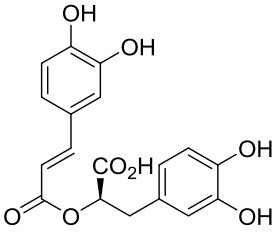


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Table 3. Ixodicidal effects of compounds isolated from *L. luisieri* extracts. Data is expressed as LD_{50} and LD_{90} values (nmol/mg of cellulose) and 95% Confidence Intervals (lower, upper).

Compound	LD_{50} (Lower-Upper)	LD_{90} (Lower-Upper)
5-methylene-2,3,4,4-tetramethylcyclopent-2-enone (**3**)	43.1 (39.0-47.8)	67.5 (61.1-76.4)
3,4,5,5-tetramethylcyclopenta-1,3-diene-1-carboxylic acid (**5**)	47.1 (40.0-53.8)	82.8 (72.6-101.2)
Methyl 3,4,5,5-tetramethylcyclopenta-1,3-diene-1-carboxylate (**5a**)	> 111.1	-
5-(hydroxymethyl)-2,3,4,4-tetramethylcyclopent-2-en-1-one (**1**)	50.6 (44.4-57.2)	83.9 (73.8-101.9)
(2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)methyl acetate (**2**)	18.9 (16.8-21.4)	31.8 (28.2-37.3)
3,3,4,5-tetramethyl-2H-pyran-2,6(3H)-dione (**6**)	20.1 (18.4-22.1)	26.0 (23.7-29.5)
2-(hydroxymethyl)-3,4,4-trimethyl-5-methylidenecyclopent-2-en-1-one (**4**)	48.0 (43.0-54.1)	77.5 (68.6-91.5)
(1R,6R,7S,10R)-10-Hydroxy-4(5)-cadinen-3-one (**9**)	> 84.7	-
Camphor (**8**)	> 131.6	-
Thymol	9.8 (6.8-11.8)	20.5 (17.7-26.2)

The presence of compounds **1** and **2** correlated with the increased activity of extracts Hx, OE0 and OE2. Compound **6** has been isolated from the Hx extract (Julio *et al.*, submitted for publication) but it could not be detected by the analytical methods used in this work (GC-MS or LC-MS). Therefore, compound **6** could also be present in these active extracts. Further research is needed to implement a suitable analytical method for **6** given its importance as a biomarker. Extraction methods increasing the concentration of **2** such as supercritical CO_2 (Julio *et al.*, 2014) and / or an efficient organic extraction of the hydrolate obtained as a residue from the EO extraction could result in optimized and /or cheap ixodixidal extracts.
The ixodicidal compounds isolated from *L. luisieri* are necrodane-type monoterpenes, with an unusual skeleton that was first reported for a series of necrodol isomers isolated from the defensive secretions of the beetle *Necrodes surinamensis* (Roach *et al.*, 1990) and later as the sex pheromone of the grape mealybug *Pseudococcus maritimus* (trans-α-necrodylisobutyrate) (Figadère *et al.*, 2007) in addition to being found in the plant kingdom only in *L. luisieri*. Monoterpenes such as limonene, limonene oxide, carvacrol, thymol and pulegone showed larvicidal and toxic effects to engorged females of *R. (B.) microplus* (Ferrarini *et al.*, 2008), (De Oliveira Monteiro *et al.*, 2010), (Ribeiro *et al.*, 2010), (Cruz *et al.*, 2013), (Ramírez *et al.*, 2013), thymol also has acaricidal activity against inmature stages of *Ambylomma cajennense* (Da Silva Mendes *et al.*, 2011) and geraniol repelled *Ixodes ricinus* (Kröber *et al.*, 2013). However, this is the first report on acaricidal effects for necrodane-type monoterpenes.
Tyramine and octopamine receptors have been proposed as molecular targets for monoterpenoids in insects and mites (Blenau *et al.*, 2012). Thymol, carvacrol and pulegone affect insect $GABA_A$ receptors (Priestley *et al.*, 2003), (Tong and Coats, 2010) and carvacrol inhibits *D. melanogaster* transient receptor potential channels (Parnas *et al.*, 2009). Therefore, the acaricidal mode of action of necrodane-type monoterpenes could be receptor mediated, but further research is needed to proof this hypothesis. These compounds may represent a new class of ixodicidal agents.

4. Conclusion

The essential oil (EO), the hexane (Hx) extract and the organic fraction (OE0) of the residual hydrolate of cultivated <u>*Lavandula luisieri*</u> showed larvicidal effects against the hard tick *Hyalomma lusitanicum*, with the hydrolate organic fraction (OE0) being the most effective extract. The presence of necrodane-type compounds in these extracts explained these effects, being (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate (**2**) and 3,3,4,5-tetramethyl-2H-pyran-2,6(3H)-dione (**6**) the most potent ones with activity levels within the range of the positive control thymol and lower volatility (M^+ 210 and 168 vs. 150 for thymol). These compounds may represent a new class of ixodicidal agents. Extraction methods increasing the concentration of **2** such as supercritical CO_2 (Julio *et al.*, 2014) or an efficient extraction of the residual hydrolate could result in optimized and/or cheap acaricidal extracts.

Acknowledgements: This work has been supported by grants CTQ2012-38219-C03-01, AGL2012-37957 (MINECO) and Villamagna SA. The authors are especially grateful to his Grace the Duke of Westminster and LF Julio to a JAE-CSIC predoctoral fellowship.

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5. CONCLUSIONES

Conclusiones

1. Los aceites esenciales de *Artemisia absinthium* en fase de pre-domesticación y obtenidos por hidrodestilación (HD) se caracterizaron por la presencia de *cis*-epoxiocimeno, crisantenol y acetato de crisantenilo. Se observaron variaciones cuantitativas en la composición del aceite esencial según el año de cosecha. Se identifica el compuesto (*Z*)-2,6-dimetilocta-5,7-dieno-2,3-diol, uno de los componentes más abundantes de algunas poblaciones.

2. Los aceites HD de *A. absinthium* en fase de pre-domesticación, ricos en *cis*-epoxiocimeno y sesquiterpenos, fueron moderadamente activos frente a *Spodoptera littoralis*, presentaron efectos antifúngicos frente a *Fusarium* sp. y efectos antiparasitarios contra *Leishmania infantum* y *Trypanosoma cruzi.*

3. La domesticación de *A. absinthium* redujo significativamente la variación de la composición química de los aceites esenciales HD y los obtenidos por arrastre de vapor en planta piloto (VP), mayor producción de biomasa y mejores rendimientos de aceite esencial. Esta población de *A. absinthium* domesticada ha dado lugar al registro de la nueva variedad ®Candial.

4. Los aceites esenciales de *A. absinthium* (®Candial) (HD y VP) se caracterizaron por la presencia de *cis*-epoxiocimeno, crisantenol, acetato de crisantenilo, *trans*-cariofileno y linalool. El compuesto (*5Z*)-2,6-dimetilocta-5,7-dien-2,3-diol solamente apareció en los extractos HD. La presencia de cineol y alcanfor correlacionó con el nivel de domesticación de *A. absinthium*.

5. El compuesto (-)-*cis*-crisantenol (**A2**) se ha identificado como el principal agente antifúngico presente en los extractos de VP de *A. absinthium* (®Candial), seguido de linalool (**A10**). Por lo tanto, el compuesto **A2** es el marcador químico de la actividad antifúngica de los extractos VP de *A. absinthium* (®Candial).
6. Los compuestos antiparasitarios de los extractos VP de *A. absinthium* (®Candial) fueron el *trans*-cariofileno y el 3,6-dihidrochamazuleno.

7. El hidrolato procedente de la obtención del extracto VP de *A. absintium* (®Candial) resultó nematicida frente al nematodo fitopatógeno *Meloidogyne javanica*, causando una alta mortalidad de los juveniles infectivos, una fuerte inhibición de la eclosión de huevos, suprimió la infectividad de los juveniles y redujo la infección de plantas de tomate. La actividad nematicida se localizó en la fracción orgánica del hidrolato.

8. El estudio químico biodirigido de la fracción orgánica nematicida del hidrolato de *A. absinthium* (®Candial) permitió la identificación de dos dioles monoterpenos conocidos: (*Z*) y (*E*)-2,6-dimetilocta-5,7-dieno-2,3-diol (**A13** y **A14**), cinco tetrahidrofuranoides (**A15**-**A18** and **A23**), cuatro tetrahidropiranoides (**A19**-**A22**) y seis tetraoles (**A24**-**A29**). Los compuestos **A16**-**A22** and **A24**-**A29** no se han descrito previamente. El compuesto **A13**

mostró efectos nematicidas contra *M. javanica* y se identifica como marcador químico de la potente actividad nematicida de este extracto.

9. Los extractos obtenidos por hidrodestilación (HD), extracción con solventes (OE) y extracción con CO_2 supercrítico (SCE) de plantas silvestres de *Lavandula luisieri* presentaron diferentes composiciones químicas y efectos antialimentarios sobre insectos.

10. La extracción SCE de *L. luisieri* silvestre mejoró la extracción de 10-hidroxi-4(5)-cadinen-3-ona (**L14**) y el ácido hexadecanoico respecto al extrtacto HD. Los extractos SCE también aumentaron los efectos antialimentarios contra *S. littoralis* y el pulgón *Myzus persicae*.

11. Los extractos de *L. luisieri* cultivada en fase de pre-domesticación presentaron efectos fitotóxicos y nematicidas, siendo el hidrolato el más activo seguido del extracto hexánico (H), etanólico (EtOH) y del aceite esencial (EO).

12. Del estudio fitoquímico de los extractos H, EO y del hidrolato (WR) de *L. luisieri* cultivada en pre-domesticación se aislaroncinco nuevos necrodanos (**L1**-**L3**, **L5** y **L11**) y dos conocidos (**L4** y **L6**), un derivado del cadinano (**L14**), alcanfor (**L13**), y los ácidos tormentico (**L15**) y ursólico (**L16**). El ácido rosmarínico (**L17**) se aisló a partir del extracto AcOEt y el nuevo producto natural **L12** del hidrolato (WR). Los compuestos **L1**, **L4**-**L6**, **L6a**, **L11** y **L14** mostraron efectos fitotóxicos con diferentes potencias. Sólo el compuesto **L11** presentó actividad nematicida, por lo tanto este nor-necrodano podría ser un compuesto cabeza de serie para la síntesis de nuevo herbicida y agentes nematicidas.

13. El análisis químico de los extractos bioactivos de *L. luisieri* pre-domesticada mostró que el EO y H se caracterizaron por la presencia de alcanfor (**L13**), seguido del necrodano **L4**. El extracto H también contenía el compuesto **L1**, cantidades más altas de **L2** que el EO y **L6**. El extracto EtOH contenía los ácidos tormentico (**L15**) y ursólico (**L16**), un derivado del cadinano (**L14**), los necrodanos **L1**, **L2**, **L5** y el ácido rosmarínico (**L17**). El extracto WR contenía **L12**, **L1**, **L5**, **L3** y **L2**, mientras que su fracción nematicida WRNL estaba enriquecida en **L12**, **L3** y **L14**. Se sugiere un efecto sinérgico tanto en la actividad nematicida como en la fitotoxica de los componentes de estos extractos.

14. El aceite esencial (EO), el extracto hexanico (H) y la fracción orgánica (OE) del hidrolato de *L. luisieri* pre-domesticada mostraron efectos larvicidas contra la garrapata *Hyalomma lusitanicum*, la fracción orgánica del hidrolato (OE) fue el extracto mas eficaz. La presencia de compuestos de tipo necrodano en estos extractos explicó estos efectos, siendo el acetato del 5-(hidroximetil)-2,3,4,4-tetrametilciclopent-2-enona (**L2**) y el 3,3,4,5-tetrametilpiran-2,6-diona (**L11***)* los más activos*.* Estos compuestos representan una nueva clase de agentes ixodicidas.

6. BIBLIOGRAFÍA

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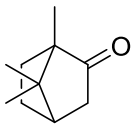
7. ANEXOS

7.1. Anexos I: Productos identificados

7.1.1. *Artemisia absinthium*



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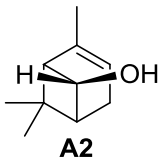

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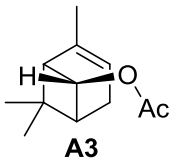

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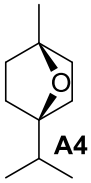

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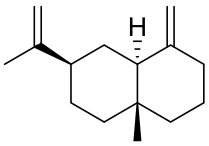

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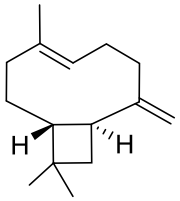

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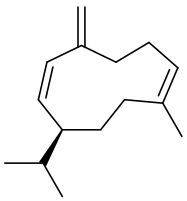

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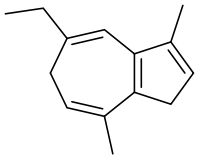

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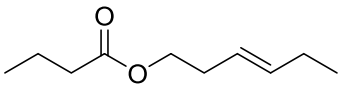

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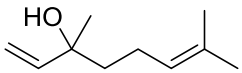

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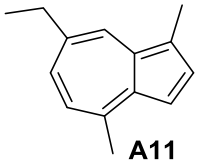

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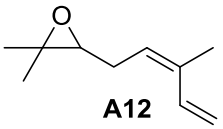

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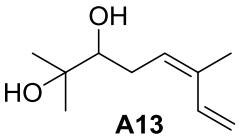

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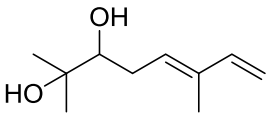

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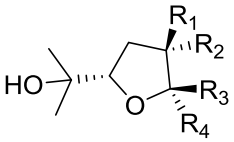
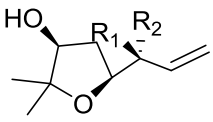
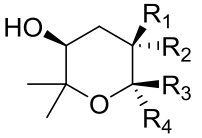
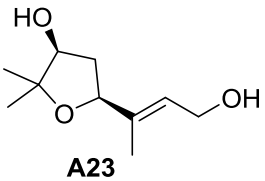






	R_1	R_2	R_3	R_4
A15	OH	H	$CH=CH_2$	CH_3
A16	H	OH	CH_3	$CH=CH_2$
A17	CH_3	OH		
A18	OH	CH_3		
A19	H	OH	CH_3	$CH=CH_2$
A20	H	OH	$CH=CH_2$	CH_3
A21	OH	H	CH_3	$CH=CH_2$
A22	OH	H	$CH=CH_2$	CH_3



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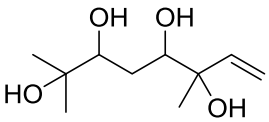
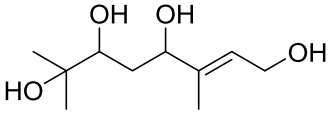
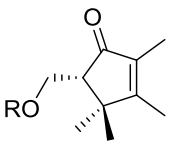


A24:**A25** and **A26**:**A27**

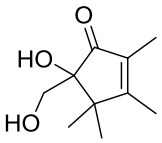


A28 and **A29**

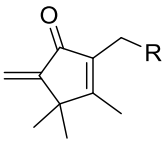
7.1.2. *Lavandula luisieri*



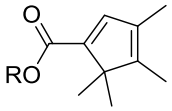
L1 R= H
L2 R= Ac



L3



L4 R= H
L5 R= OH



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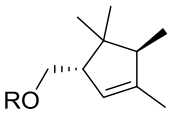
L6a R= Me



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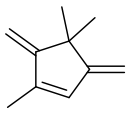
L8 R = Ac



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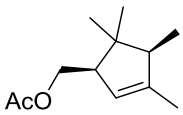

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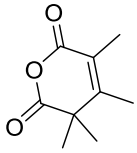

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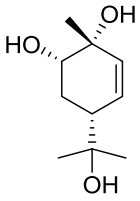

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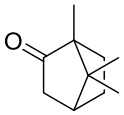

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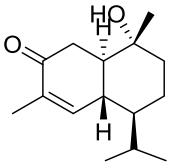

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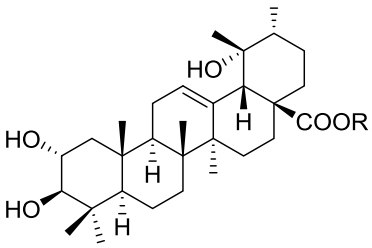

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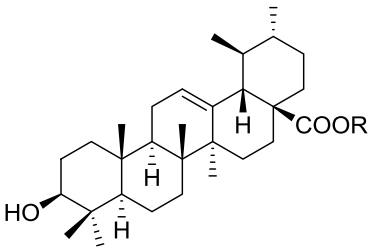
L15a R = Me



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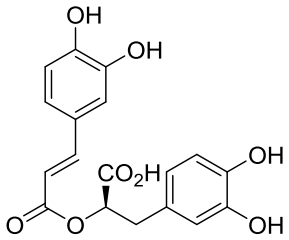
L16a R = Me



L17

7.2. Anexos II: Espectros de RMN (^{1}H y ^{13}C) de los compuestos aislados

7.2.1. *Artemisia absinthium*.

Chemical characterization of the nematicidal hydrolate from Artemisia absinthium.

Luis F. Julio, Azucena González-Coloma, Jesus Burillo, María Fe Andrés-Yeves, Carmen E. Diaz

Supporting information

List of NMR Spectrum

Fig (1) ^{1}H-NMR spectrum of compound **3** ($CDCl_3$, 500 MHz)
Fig (2) ^{13}C-NMR spectrum of compound **3** ($CDCl_3$, 125 MHz)
Fig (3) ^{1}H-NMR spectrum of compound **4** ($CDCl_3$, 500 MHz)
Fig (4) ^{13}C-NMR spectrum of compound **4** ($CDCl_3$, 125 MHz)
Fig (5) ^{1}H-NMR spectrum of compound **5** ($CDCl_3$, 500 MHz)
Fig (6) ^{13}C-NMR spectrum of compound **5** ($CDCl_3$, 125 MHz)
Fig (7) ^{1}H-NMR spectrum of compound **6** ($CDCl_3$, 500 MHz)
Fig (8) ^{13}C-NMR spectrum of compound **6** ($CDCl_3$, 125 MHz)
Fig (9) ^{1}H-NMR spectrum of compound **7** ($CDCl_3$, 500 MHz)
Fig (10) ^{13}C-NMR spectrum of compound **7** ($CDCl_3$, 125 MHz)
Fig (11) ^{1}H-NMR spectrum of compound **8** ($CDCl_3$, 500 MHz)
Fig (12) ^{13}C-NMR spectrum of compound **8** ($CDCl_3$, 125 MHz)
Fig (13) ^{1}H-NMR spectrum of compound **9** ($CDCl_3$, 500 MHz)
Fig (14) ^{13}C-NMR spectrum of compound **9** ($CDCl_3$, 125 MHz)
Fig (15) ^{1}H-NMR spectrum of compound **10** ($CDCl_3$, 500 MHz)
Fig (16) ^{13}C-NMR spectrum of compound **10** ($CDCl_3$, 125 MHz)
Fig (17) ^{1}H-NMR spectrum of compound **11** ($CDCl_3$, 500 MHz)
Fig (18) ^{13}C-NMR spectrum of compound **11** ($CDCl_3$, 125 MHz)
Fig (19) ^{1}H-NMR spectrum of compound **12:13** ($CDCl_3$, 500 MHz)
Fig (20) ^{13}C-NMR spectrum of compound **12:13** ($CDCl_3$, 125 MHz)
Fig (21) ^{1}H-NMR spectrum of compound **14:15** (CD_3OD, 500 MHz)
Fig (22) ^{13}C-NMR spectrum of compound **14:15** (CD_3OD, 125 MHz)
Fig (23) ^{1}H-NMR spectrum of compound **16** (CD_3OD, 500 MHz)
Fig (24) ^{13}C-NMR spectrum of compound **16** (CD_3OD, 125 MHz)
Fig (25) ^{1}H-NMR spectrum of compound **17** (CD_3OD, 500 MHz)
Fig (26) ^{13}C-NMR spectrum of compound **17** (CD_3OD, 125 MHz)

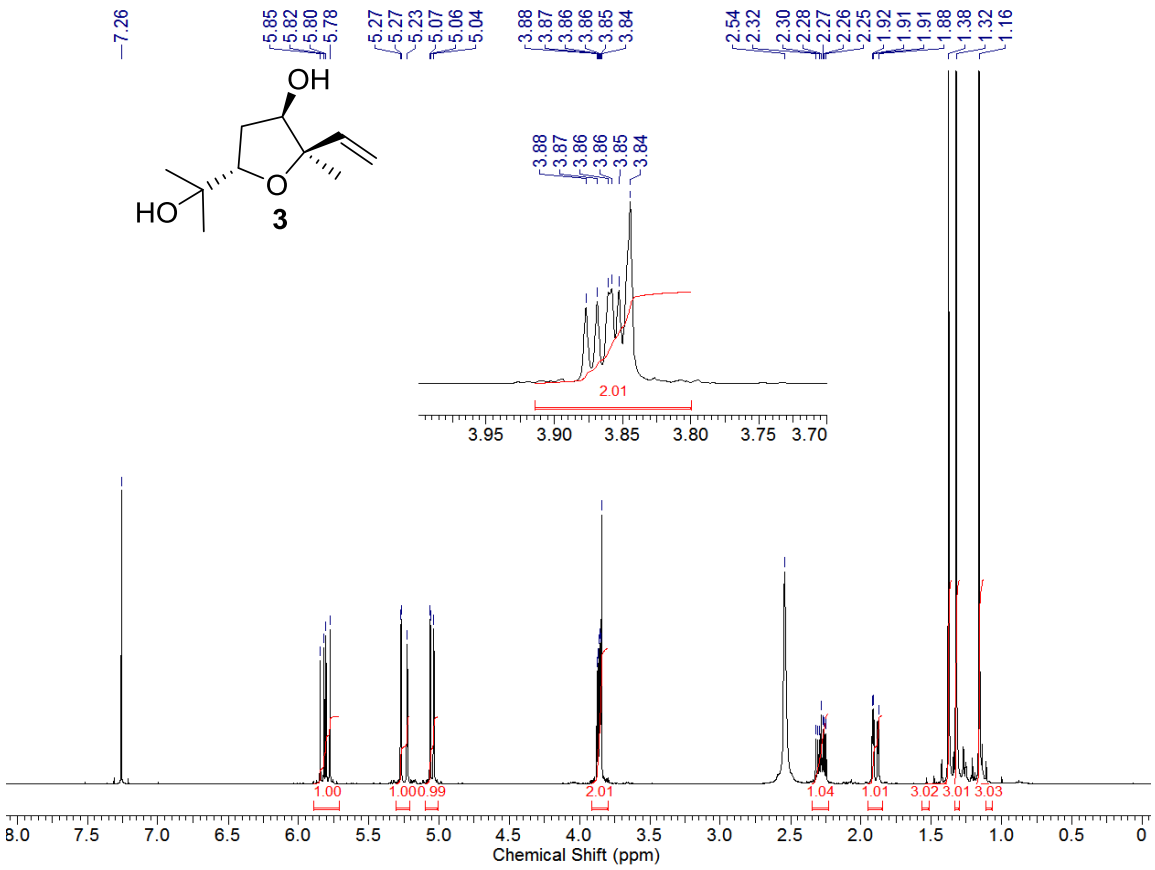


Figure 1: 1H-NMR spectrum of compound 3 (CDCl3, 500 MHz)

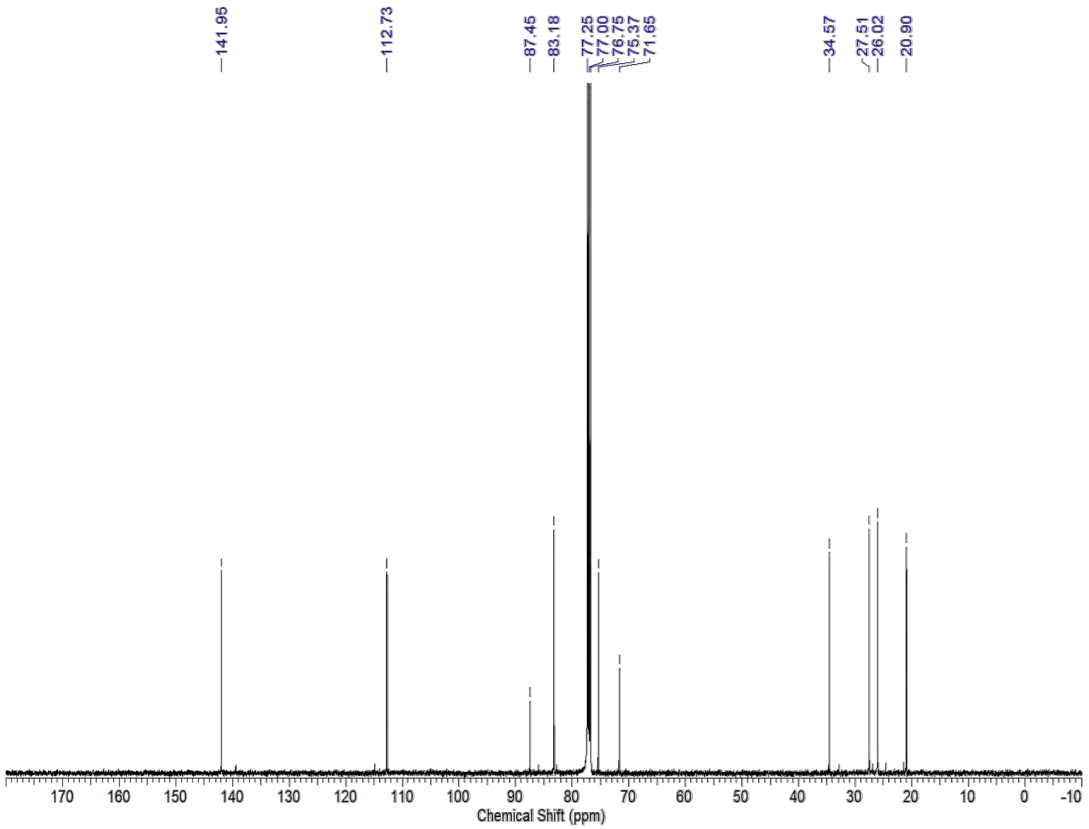


Figure 2: 13C-NMR spectrum of compound 3 (CDCl3, 125 MHz)

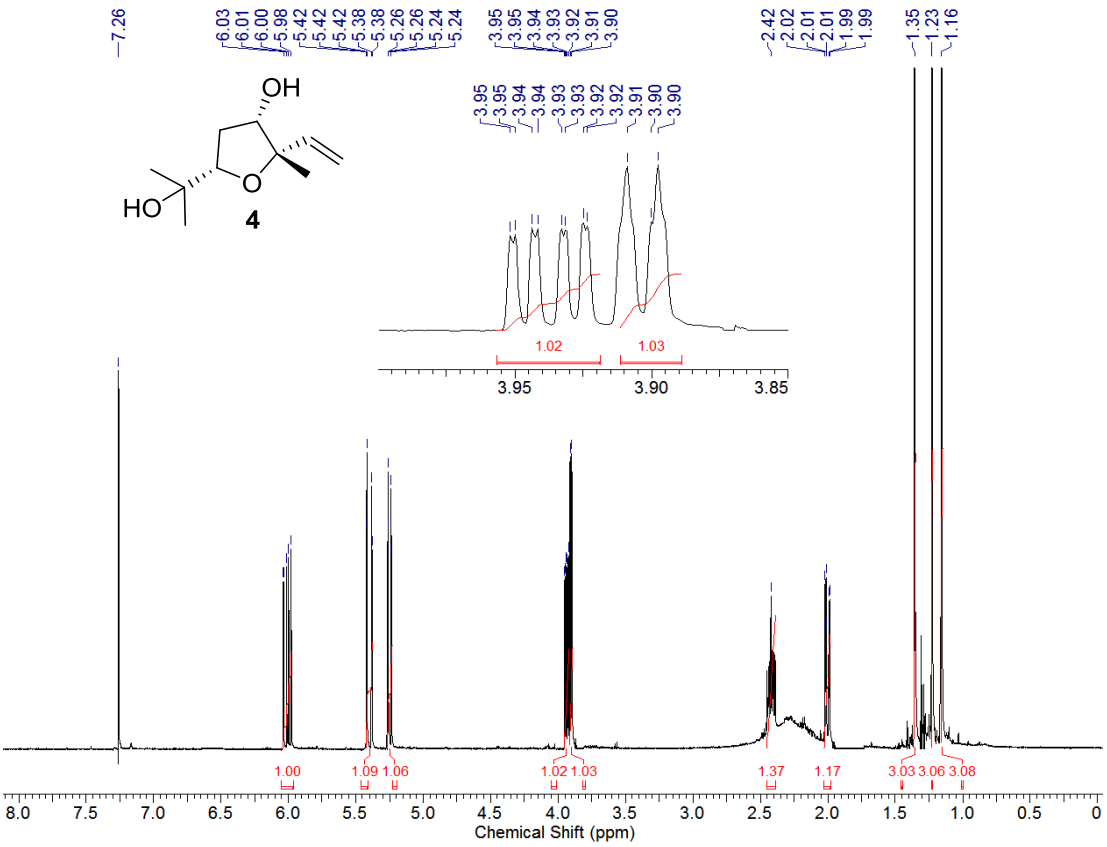


Figure 3: 1H-NMR spectrum of compound 4 (CDCl3, 500 MHz)

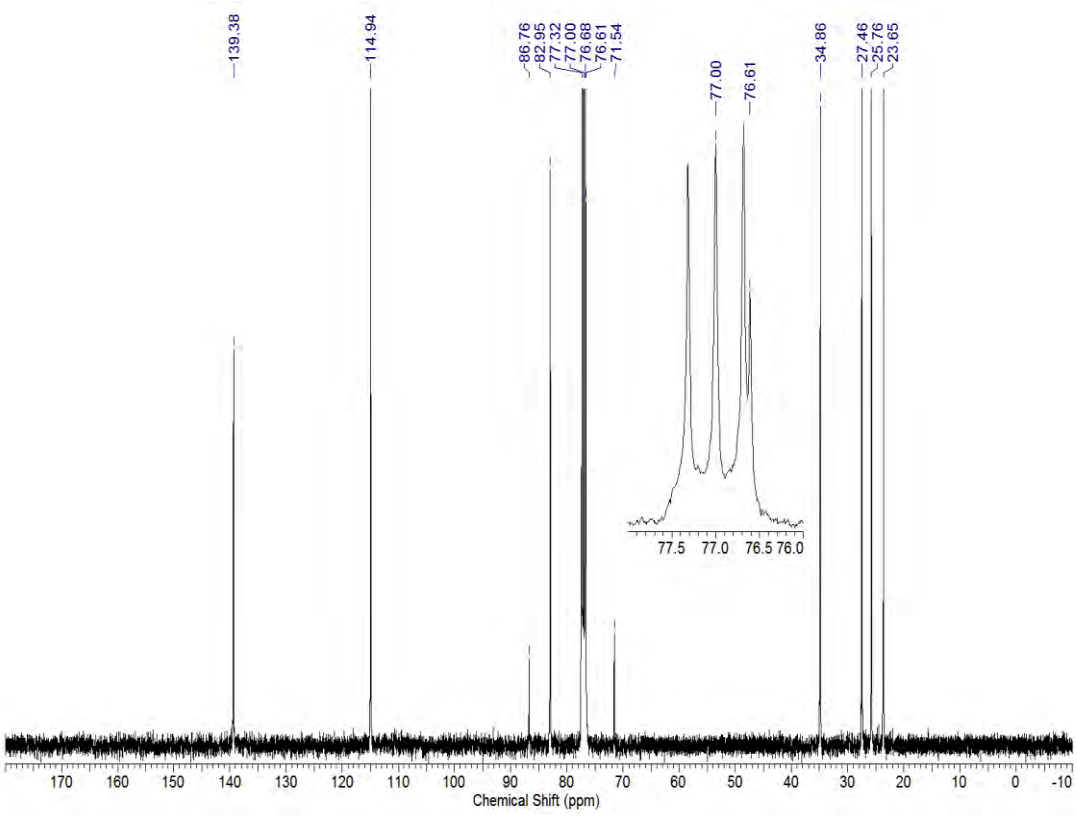


Figure 4: 13C-NMR spectrum of compound 4 (CDCl3, 125 MHz)

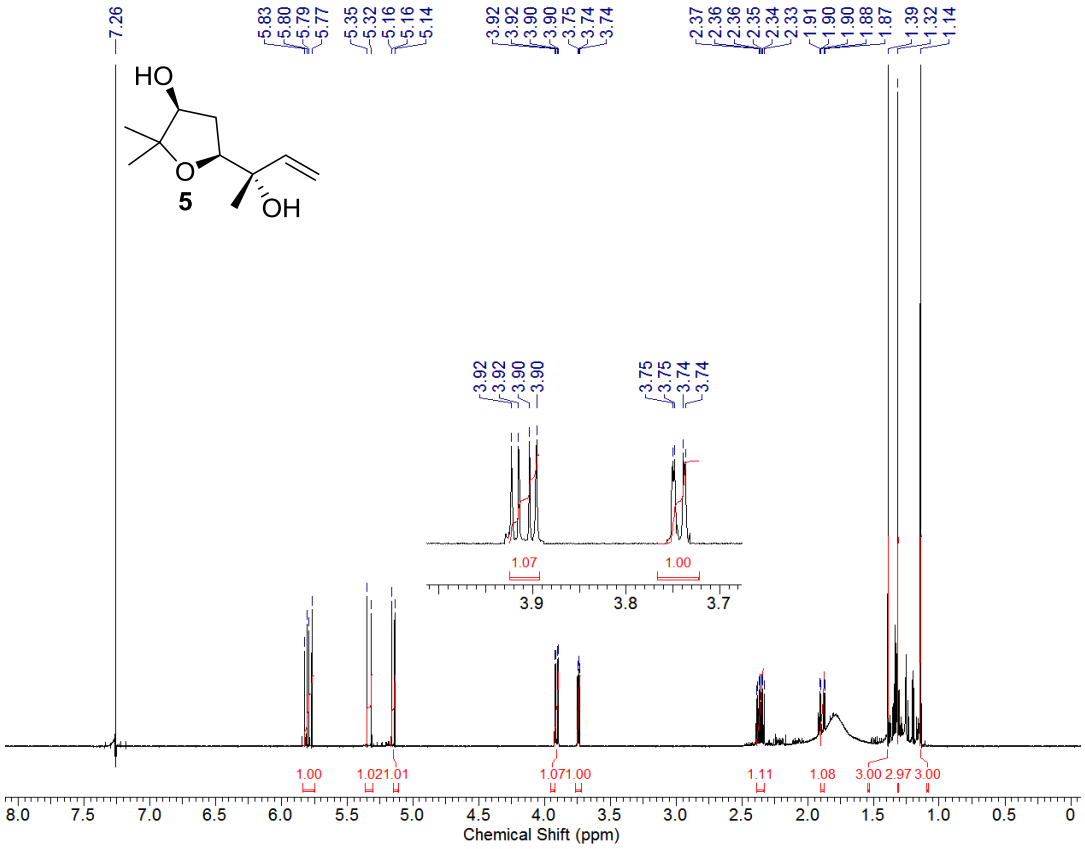


Figure 5: ^{1}H-NMR spectrum of compound **5** ($CDCl_3$, 500 MHz)

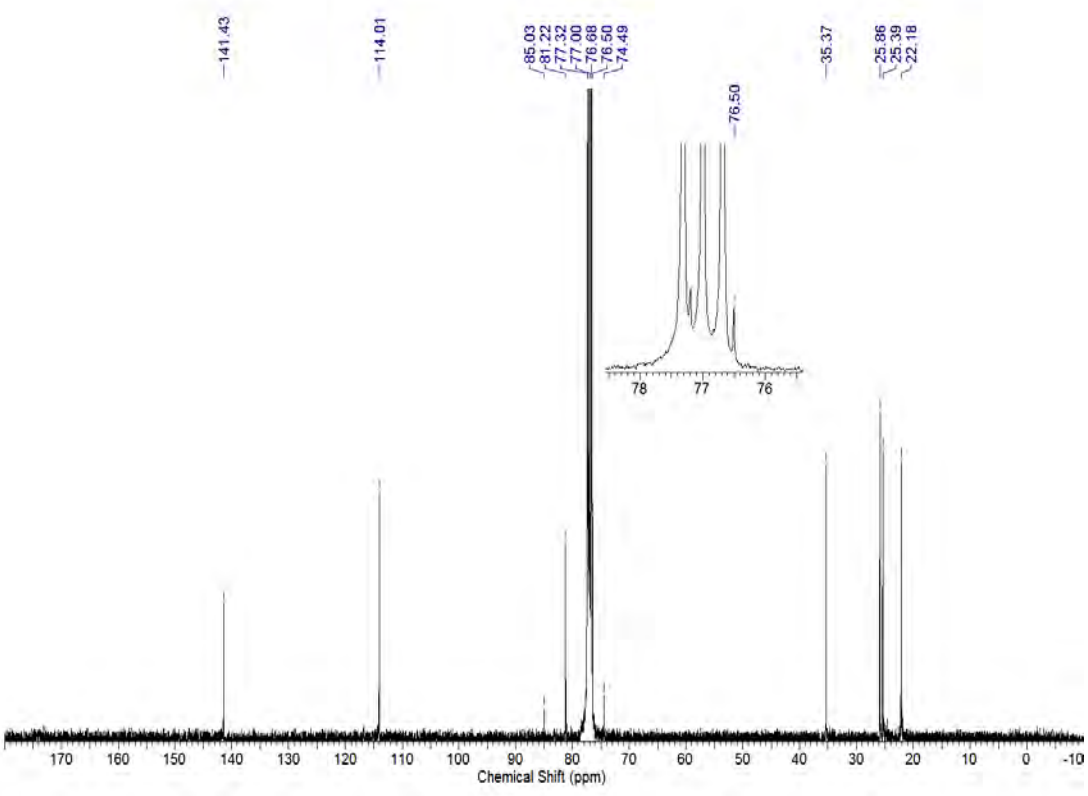


Figure: 6 ^{13}C-NMR spectrum of compound **5** ($CDCl_3$, 125 MHz)

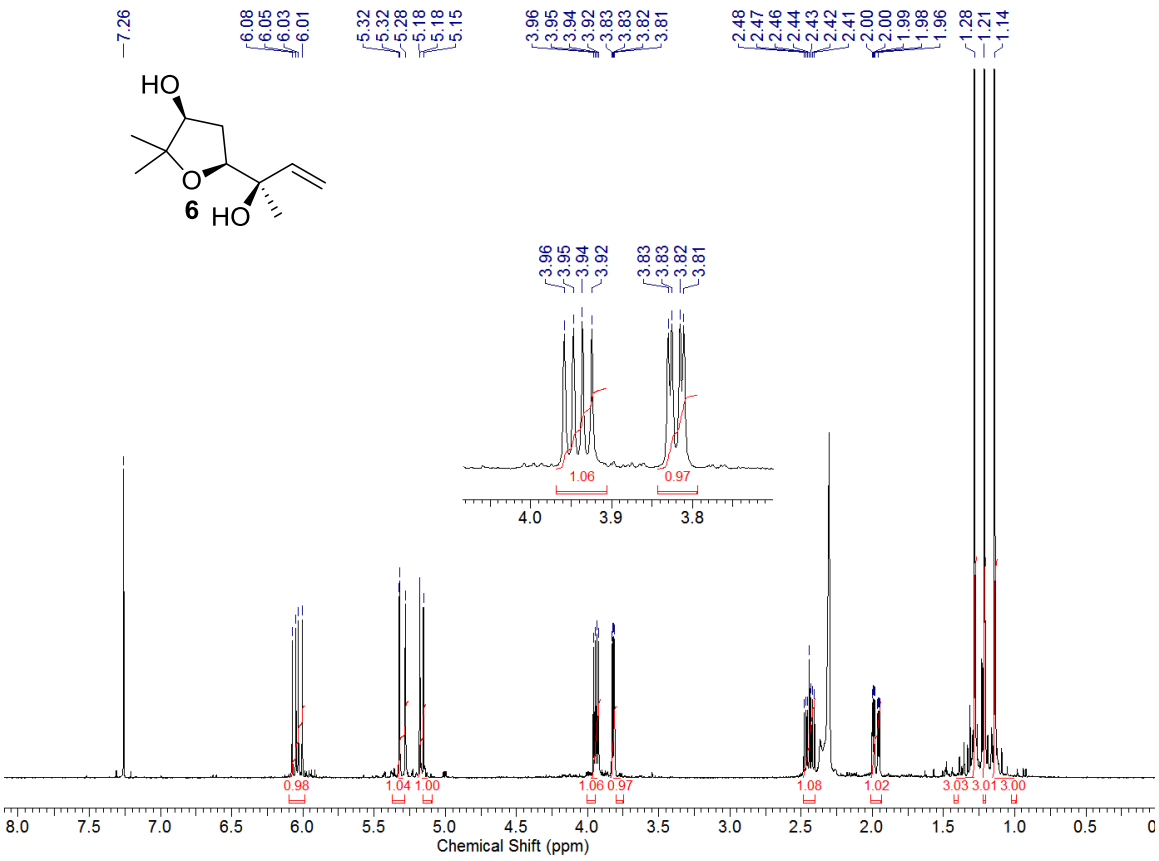


Figure 7 ^{1}H-NMR spectrum of compound **6** ($CDCl_3$, 500 MHz)

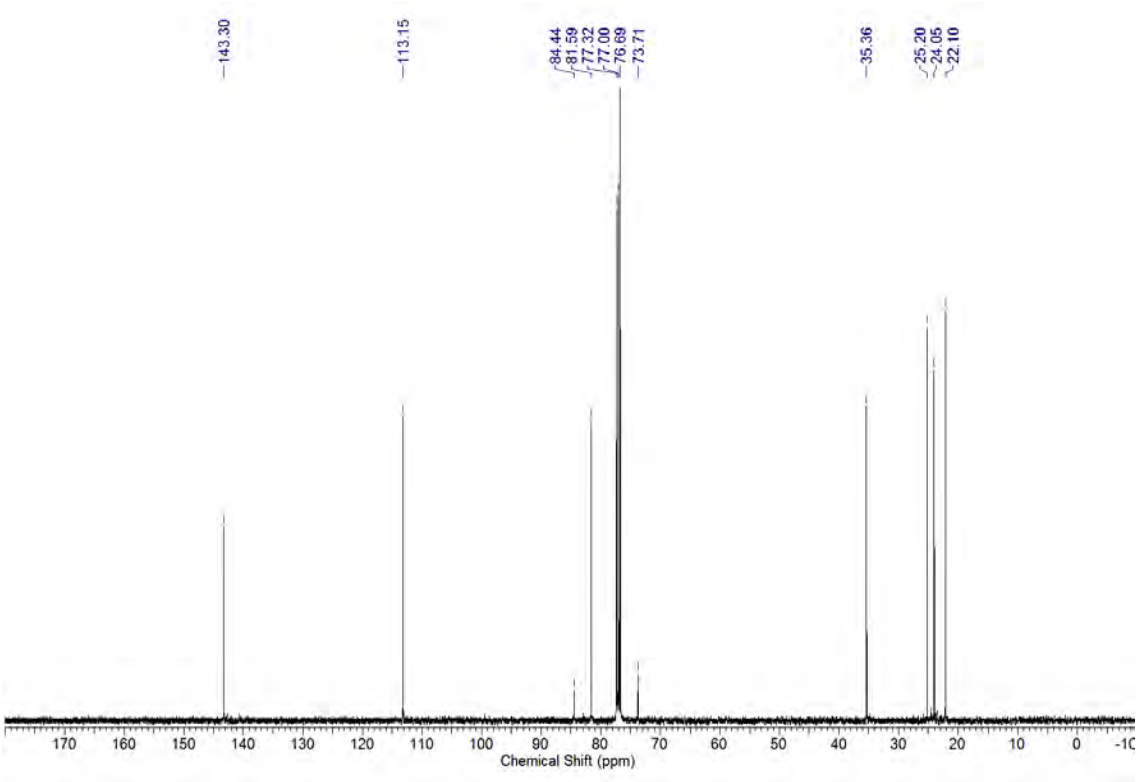


Figure 8 ^{13}C-NMR spectrum of compound **6** ($CDCl_3$, 125 MHz)

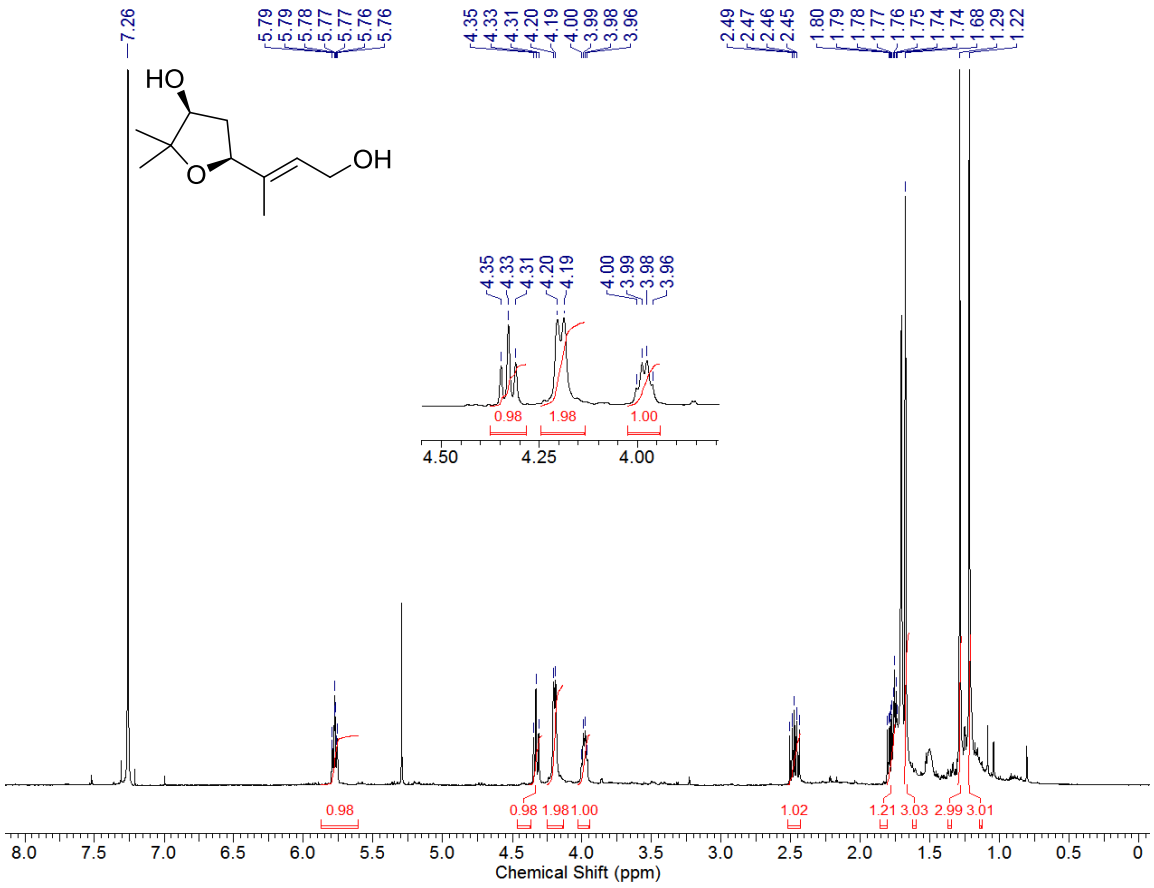


Figure 9 ^{1}H-NMR spectrum of compound **7** ($CDCl_3$, 500 MHz)

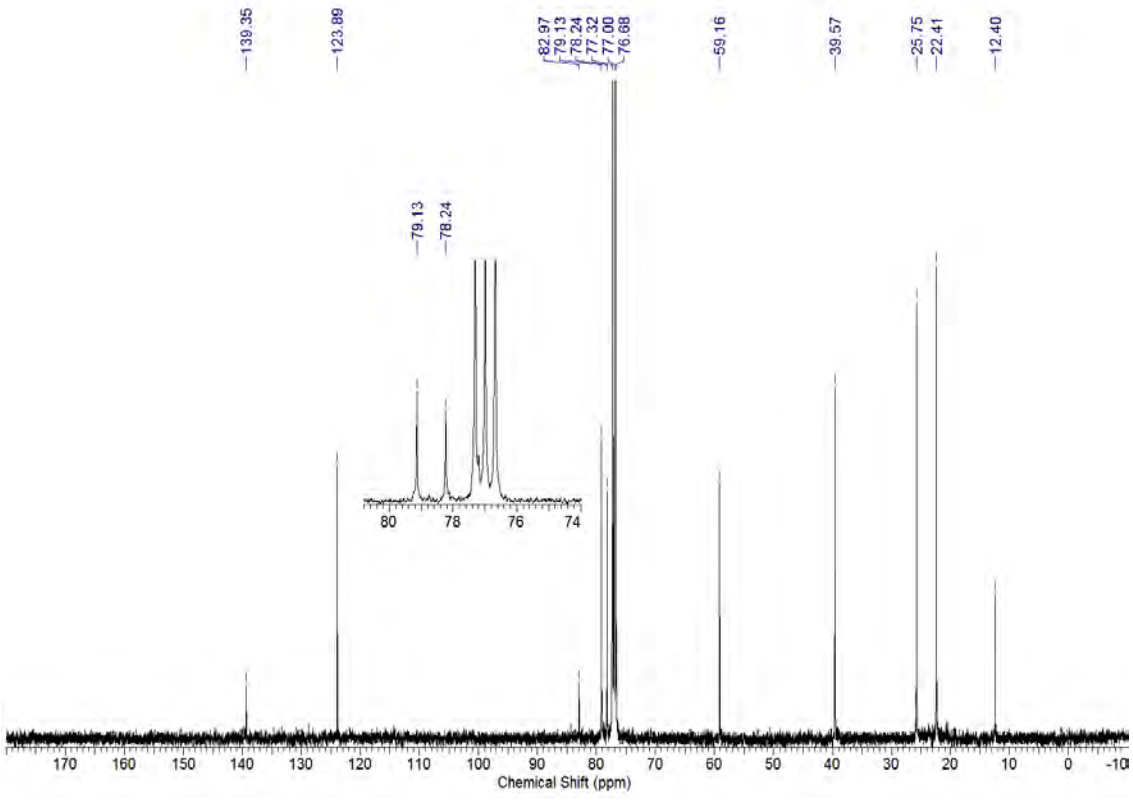


Figure 10 ^{13}C-NMR spectrum of compound **7** ($CDCl_3$, 125 MHz)

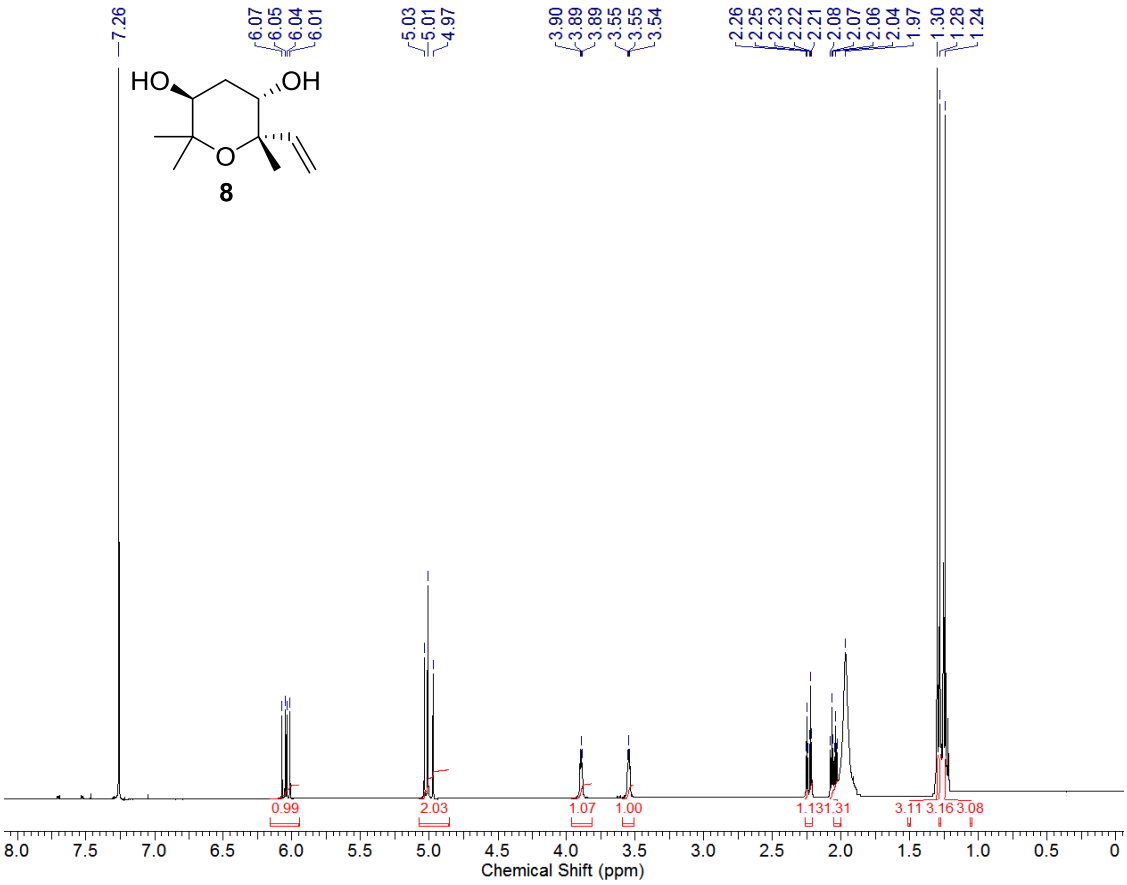


Figure 11 ^{1}H-NMR spectrum of compound **8** ($CDCl_3$, 500 MHz)

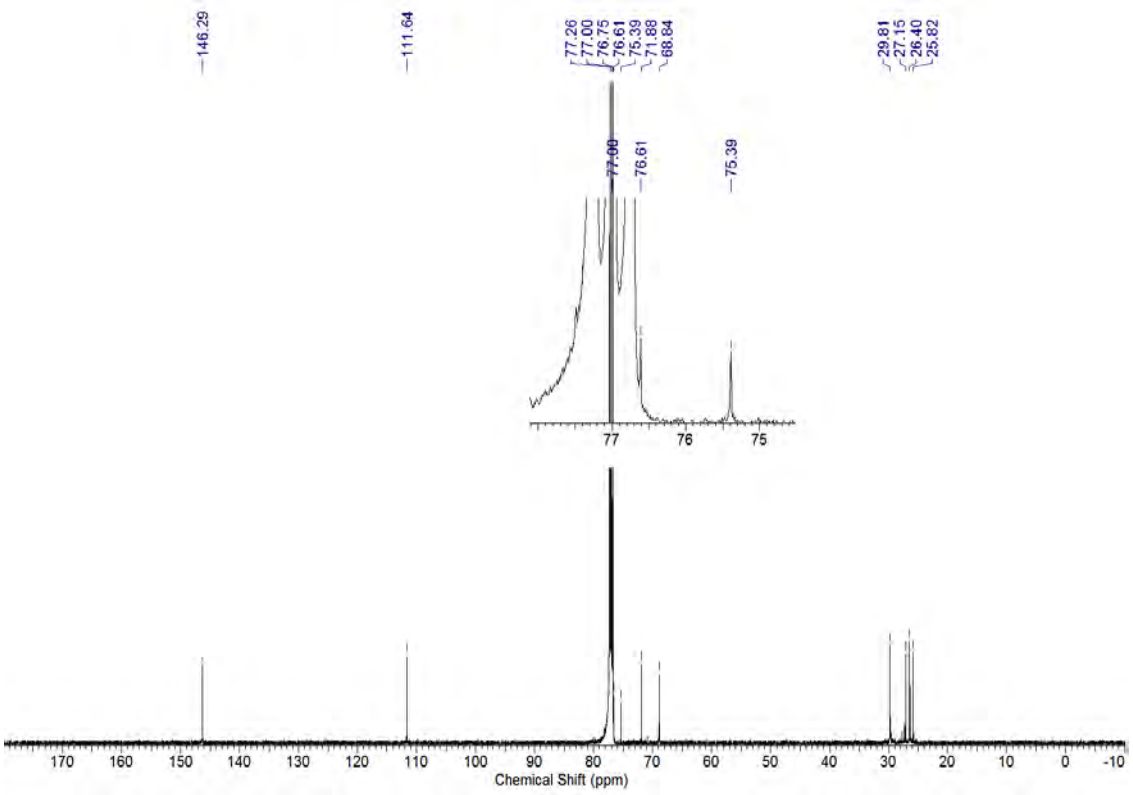


Figure 12 ^{13}C-NMR spectrum of compound **8** ($CDCl_3$, 125 MHz)

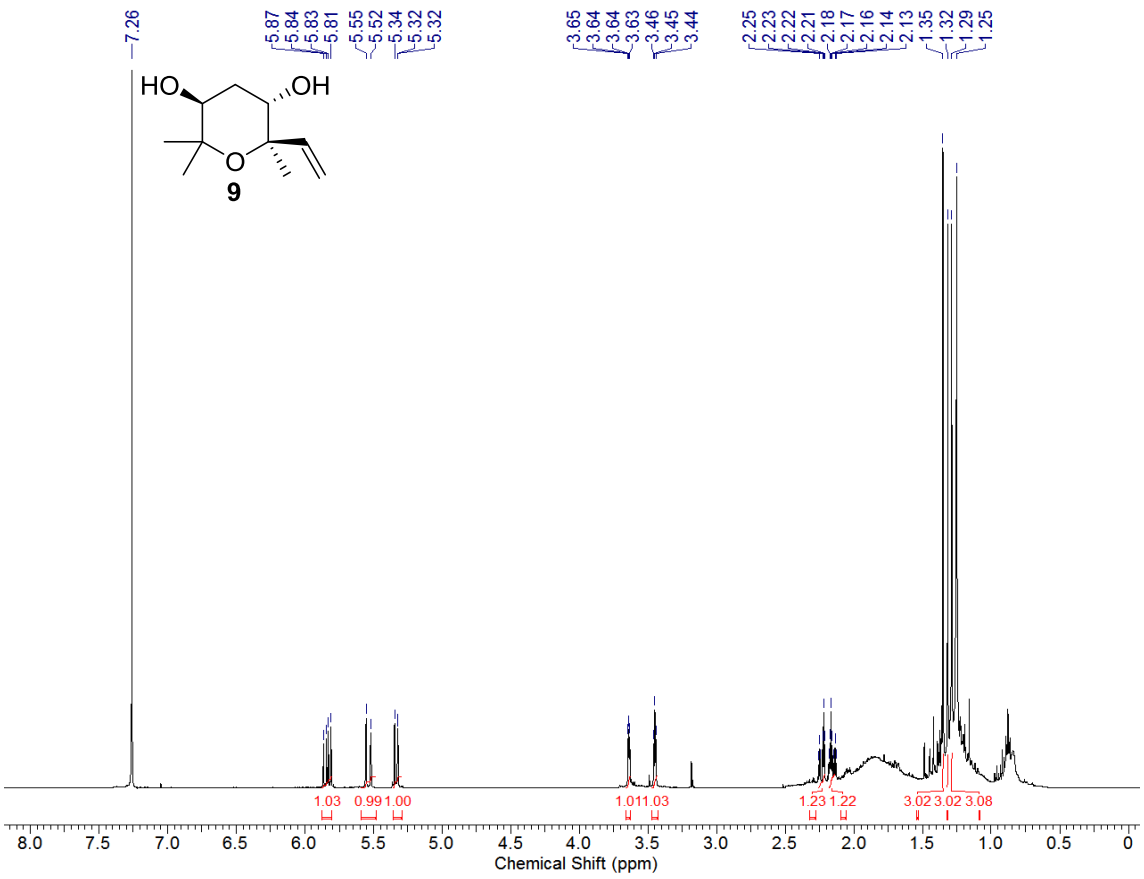


Figure 13 ^{1}H-NMR spectrum of compound **9** ($CDCl_3$, 500 MHz)

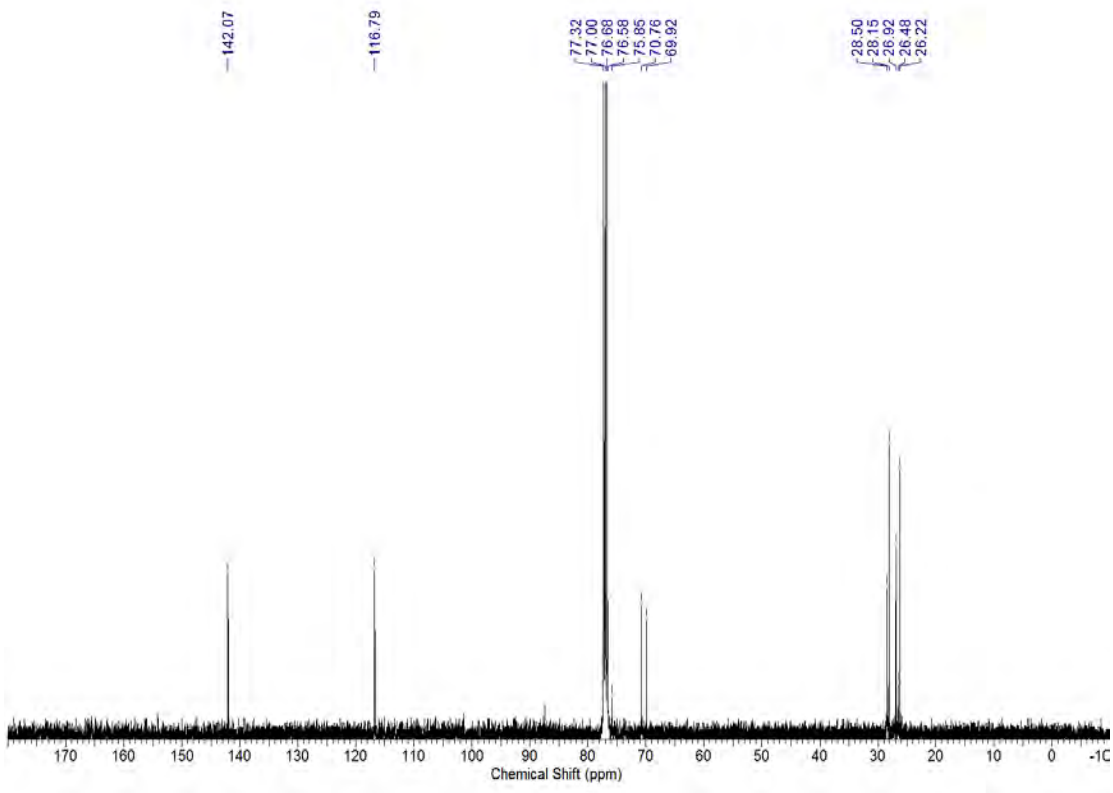


Figure 14 ^{13}C-NMR spectrum of compound **9** ($CDCl_3$, 125 MHz)

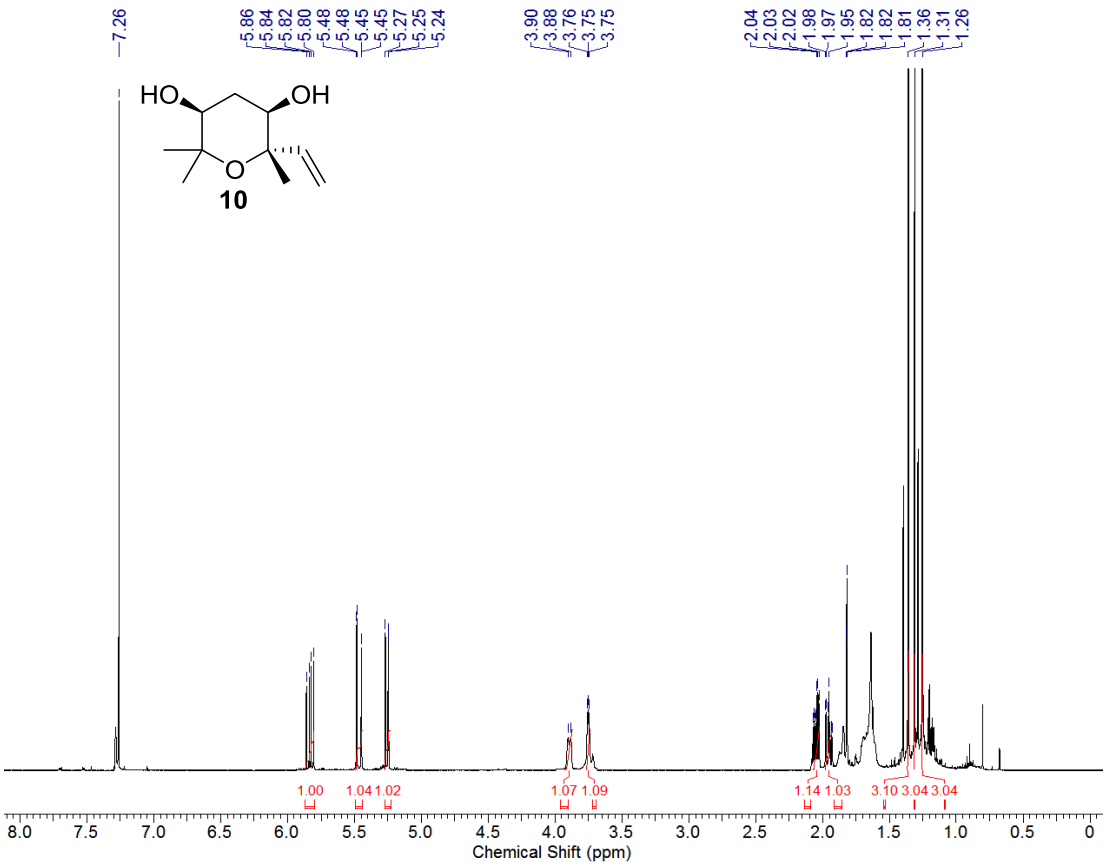


Figure 15 ^{1}H-NMR spectrum of compound **10** ($CDCl_3$, 500 MHz)

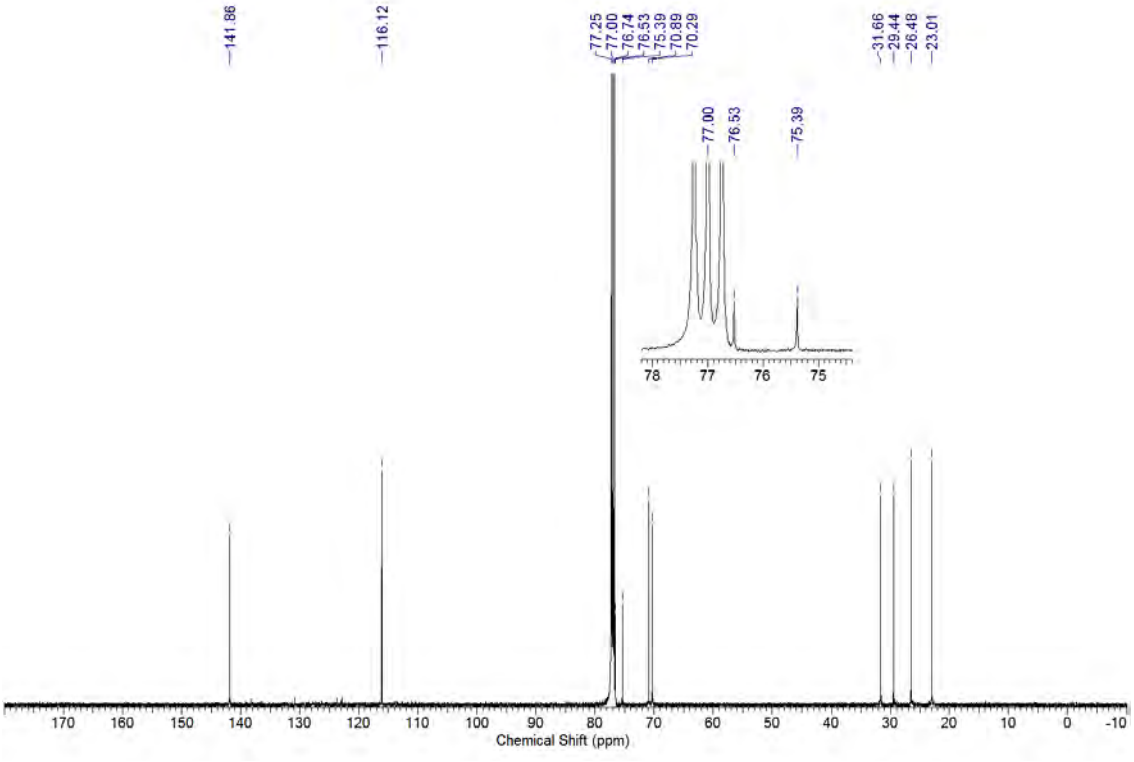


Figure 16 ^{13}C-NMR spectrum of compound **10** ($CDCl_3$, 125 MHz)

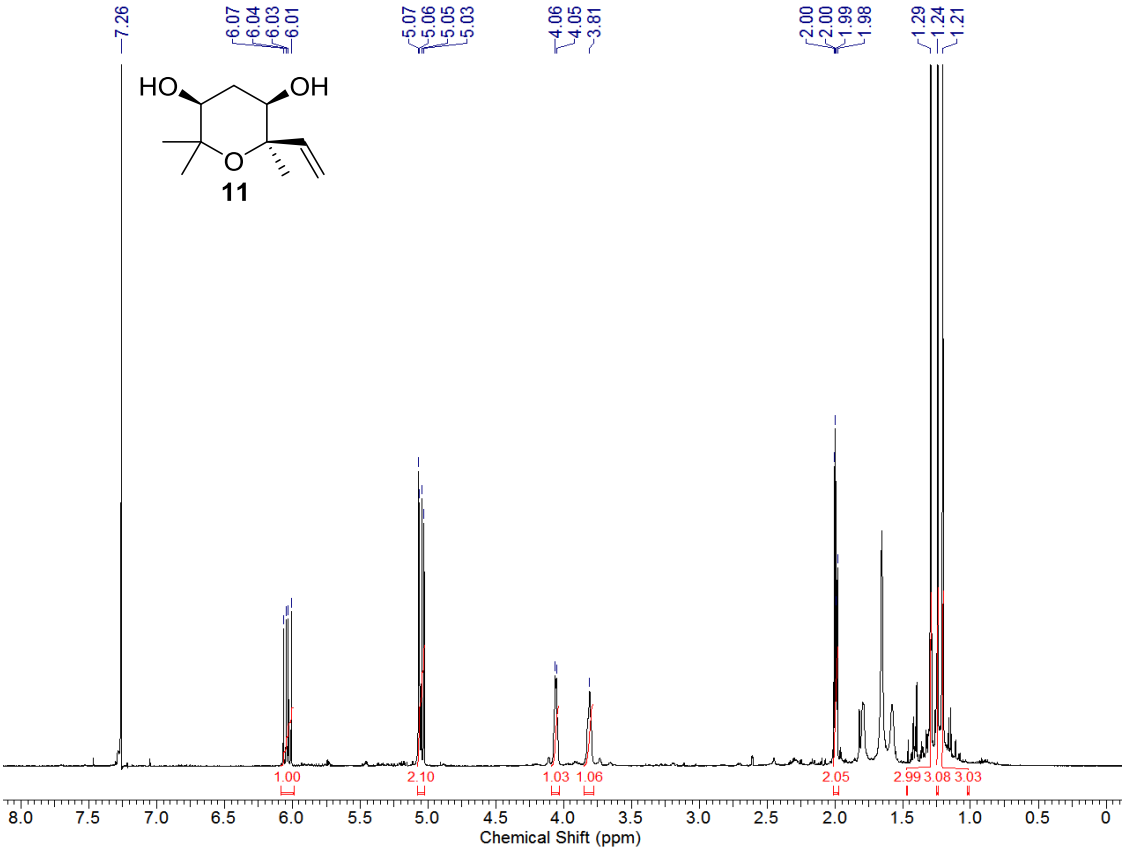


Figure 17 ^{1}H-NMR spectrum of compound **11** ($CDCl_3$, 500 MHz)

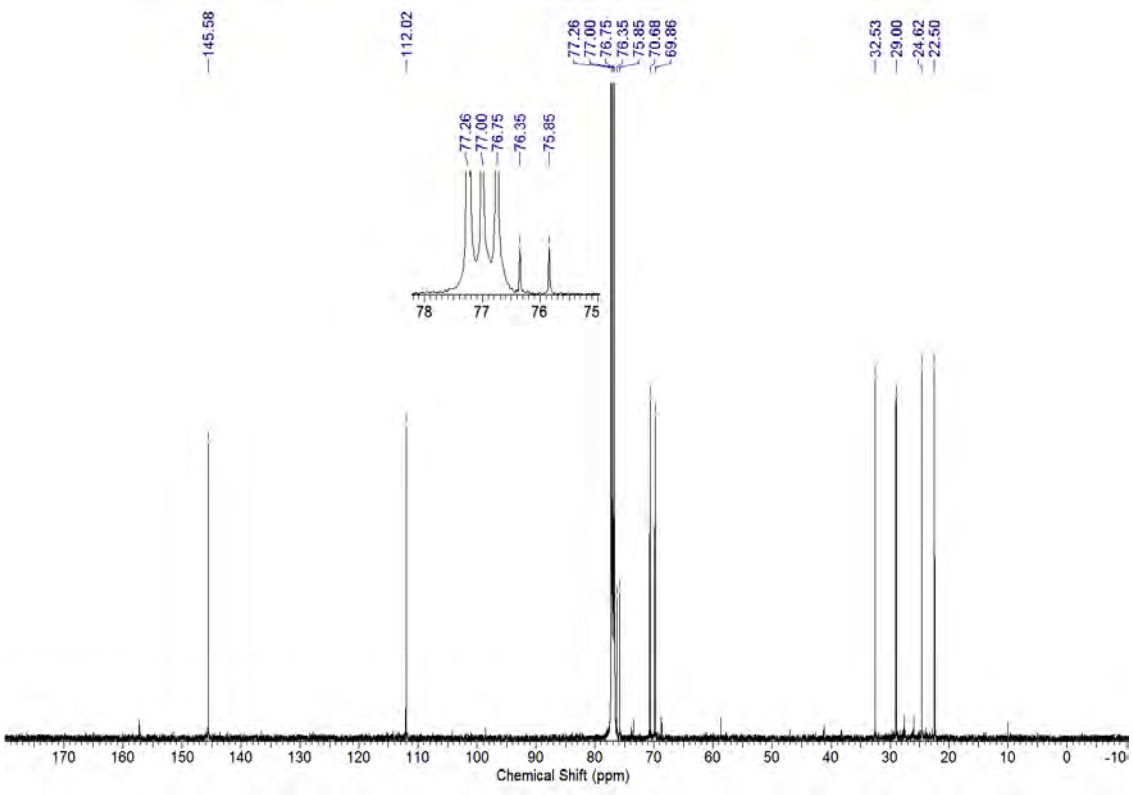


Figure 18 ^{13}C-NMR spectrum of compound **11** ($CDCl_3$, 125 MHz)

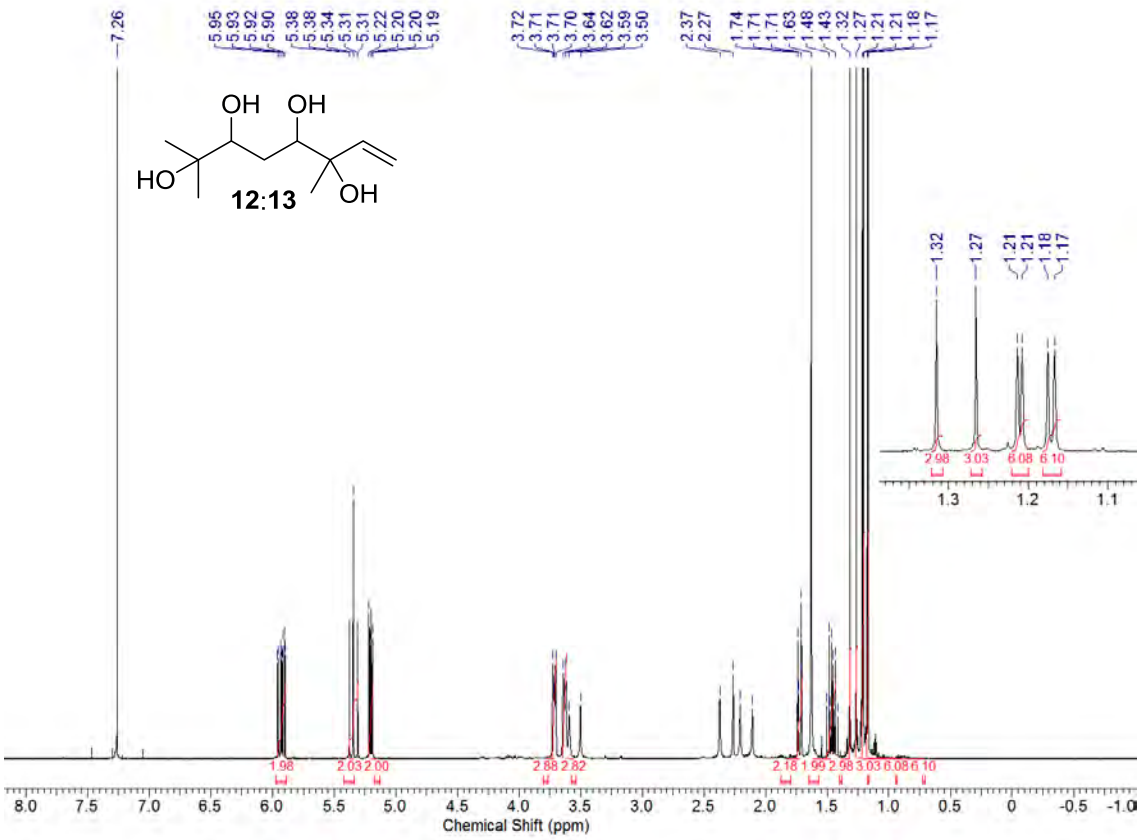


Figure 19 ^{1}H-NMR spectrum of compound **12:13** ($CDCl_3$, 500 MHz)

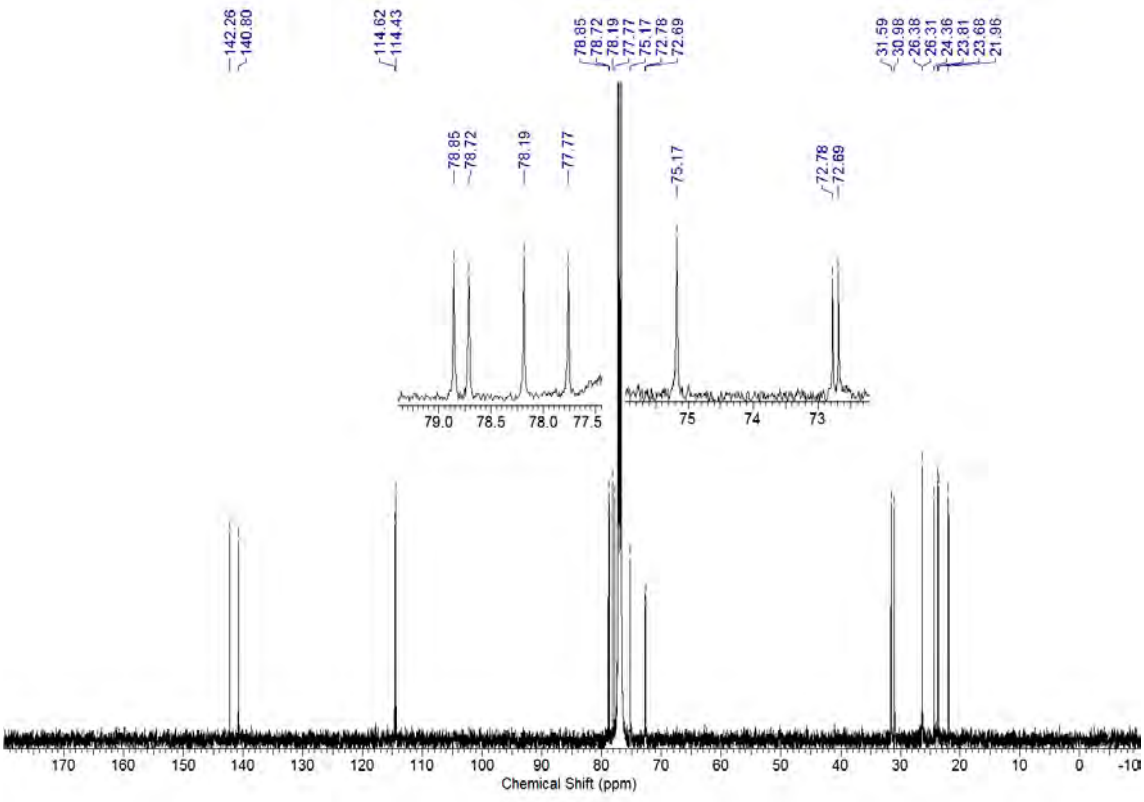


Figure 20 ^{13}C-NMR spectrum of compound **12:13** ($CDCl_3$, 125 MHz)

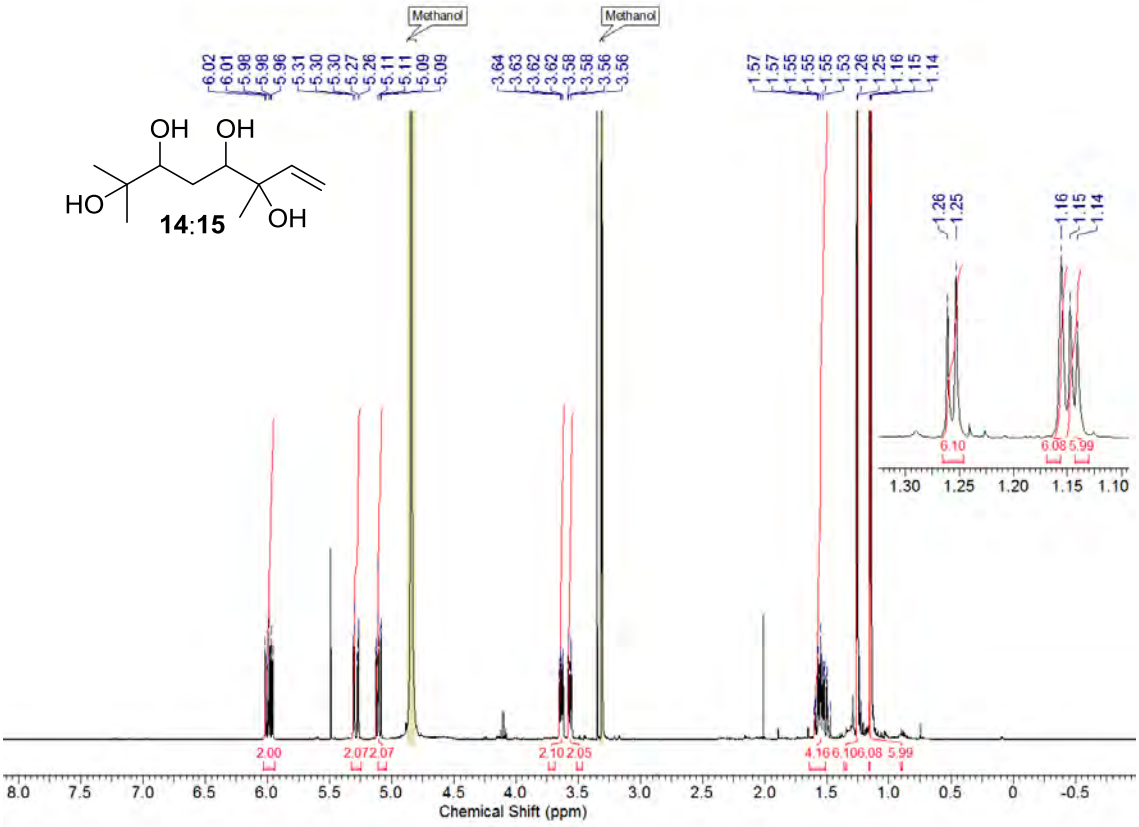


Figure 21 ^{1}H-NMR spectrum of compound **14:15** (CD_3OD, 500 MHz)

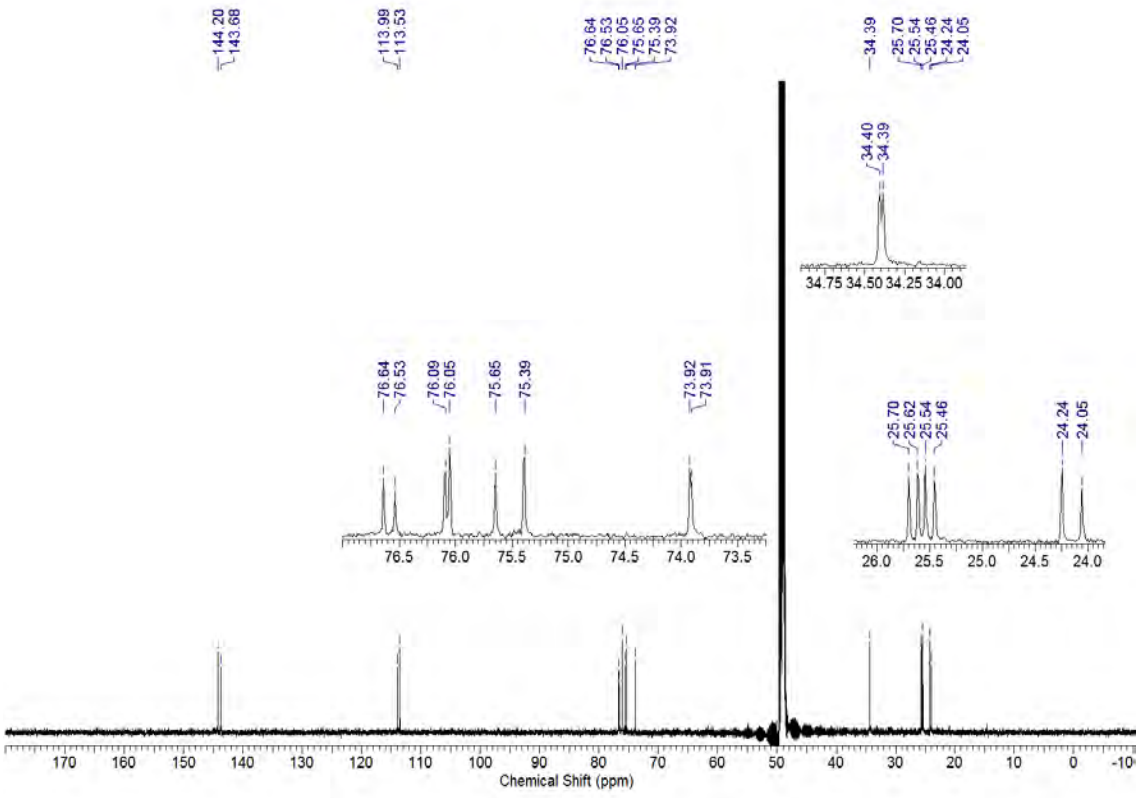


Figure 22 ^{13}C-NMR spectrum of compound **14:15** (CD_3OD, 125 MHz)

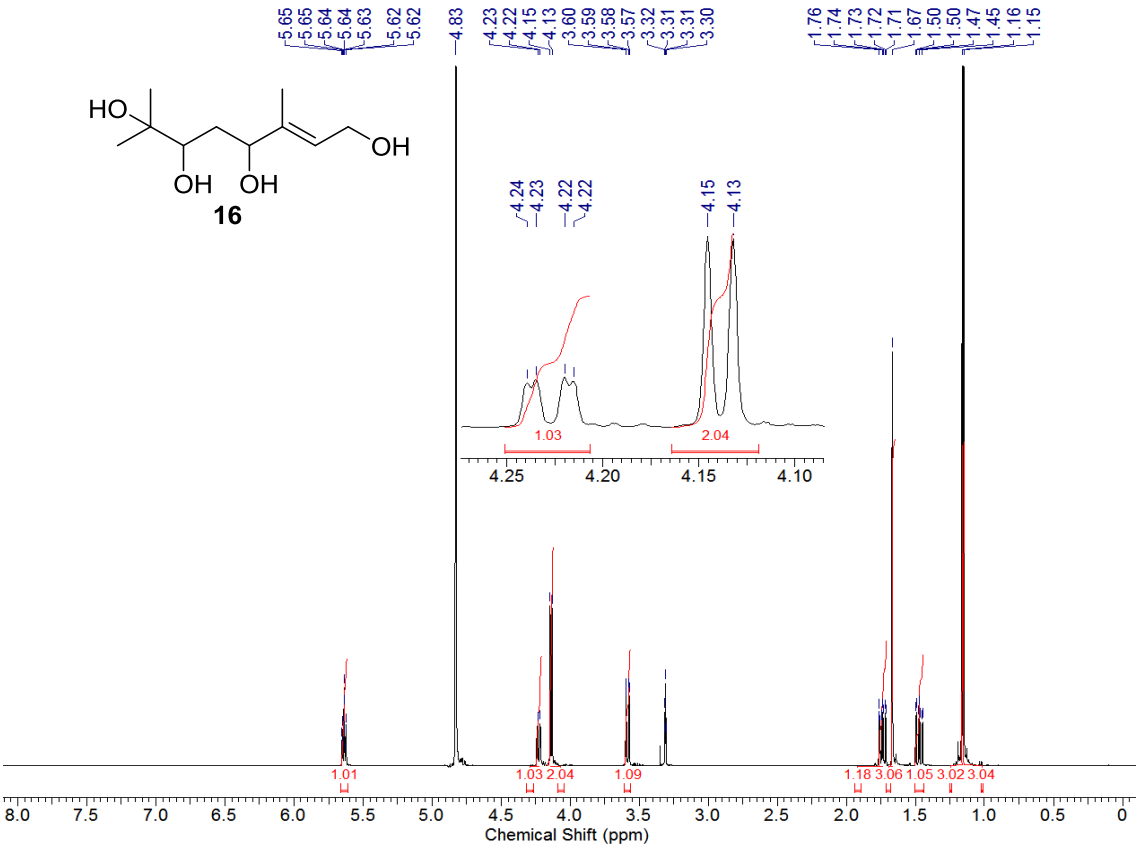


Figure 23 ^{1}H-NMR spectrum of compound **16** (CD_3OD, 500 MHz)

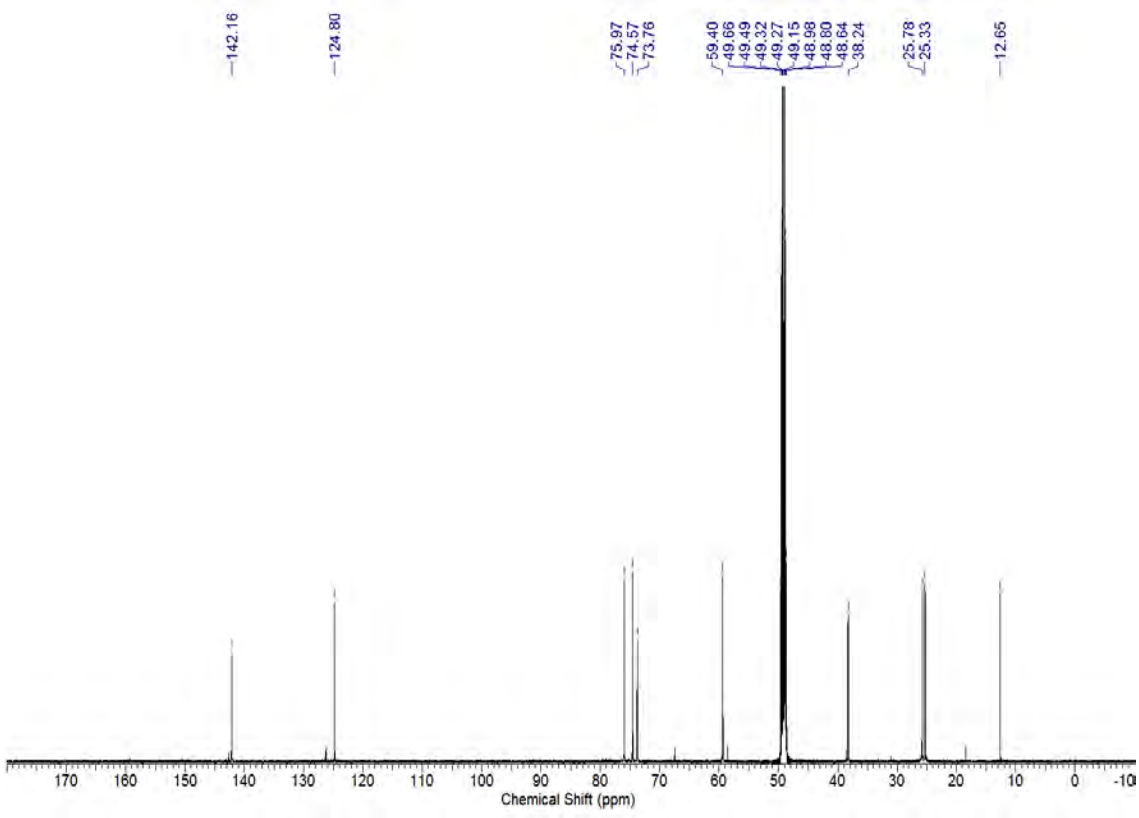


Figure 24 ^{13}C-NMR spectrum of compound **16** (CD_3OD, 125 MHz)

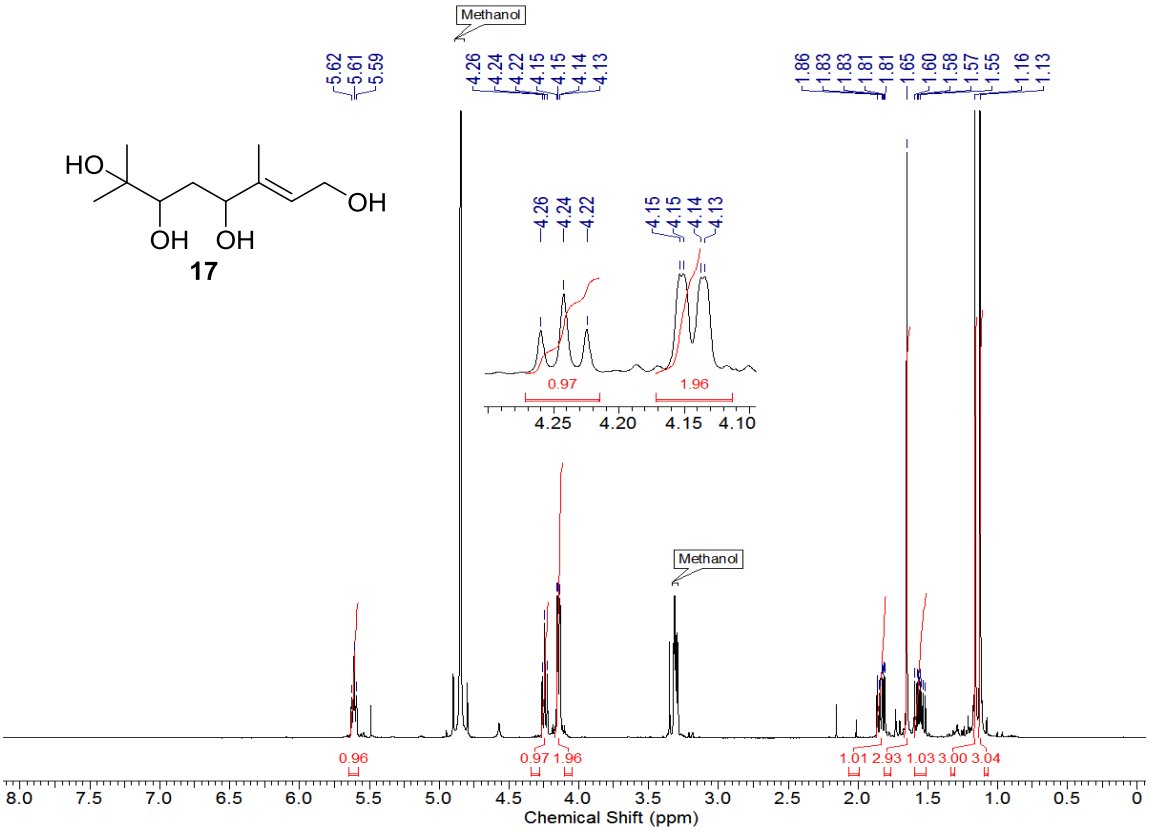


Figure 25 ^{1}H-NMR spectrum of compound **17** (CD_3OD, 500 MHz)

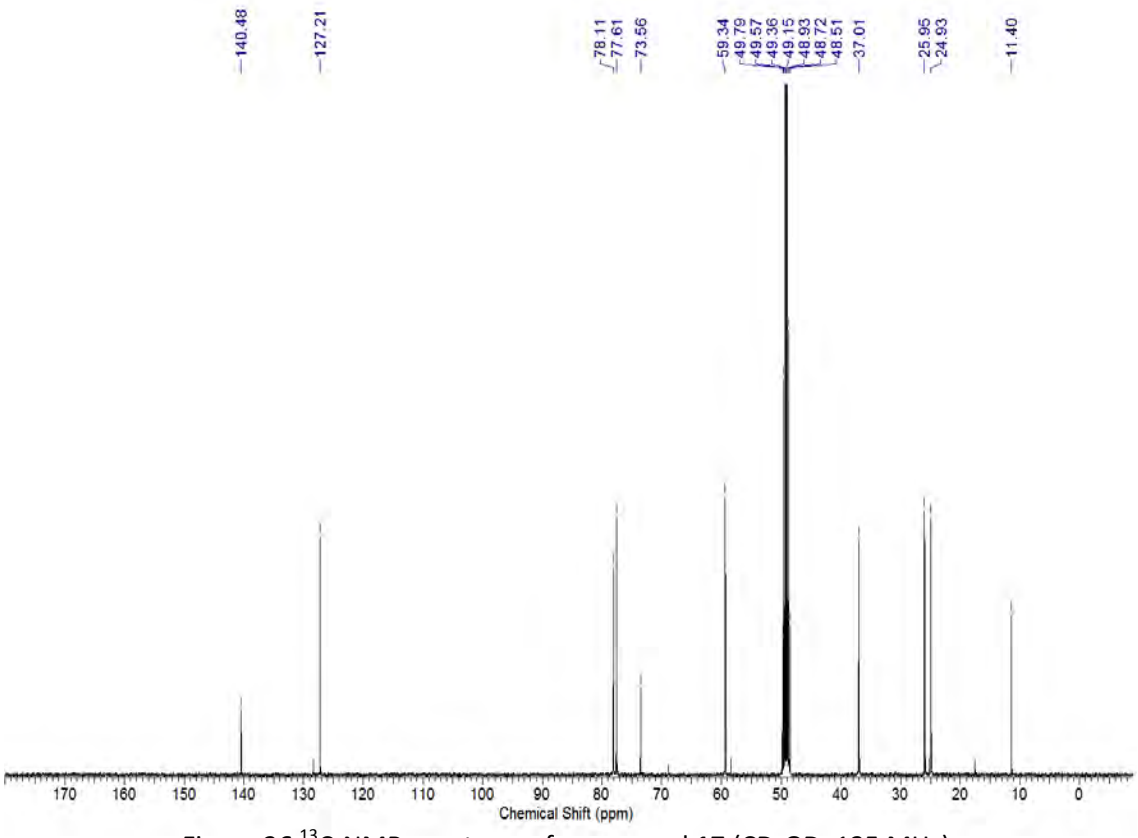


Figure 26 ^{13}C-NMR spectrum of compound **17** (CD_3OD, 125 MHz)

7.2.2. *Lavandula luisieri*.

Phytotoxic and nematicidal components of Lavandula luisieri

Luis F. Julio, Alejandro F. Barrero, M. Mar Herrador del Pino, Jesús F. Arteaga, Jesús Burillo, Maria Fe Andres, Carmen E. Díaz and Azucena González-Coloma

Supporting information

List of Supporting Information

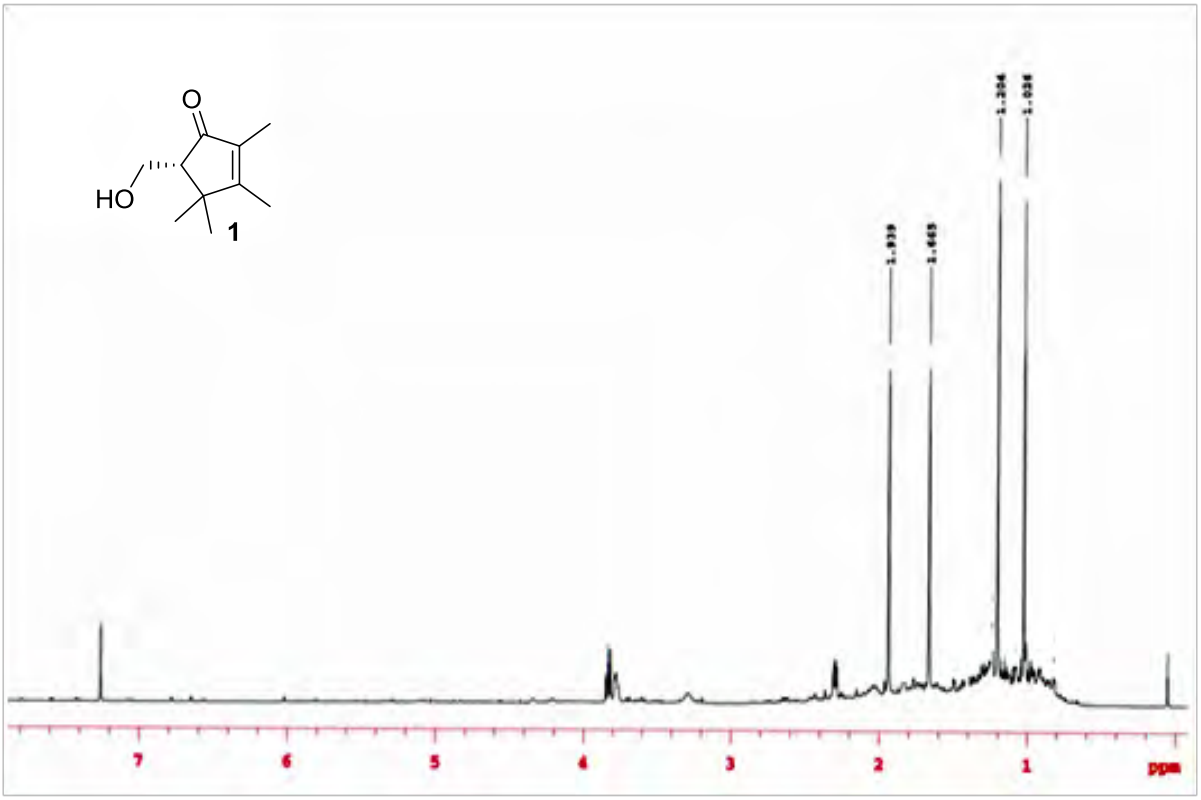


Figure 1: 1H-NMR spectrum of compound 1 (CDCl3, 500 MHz)

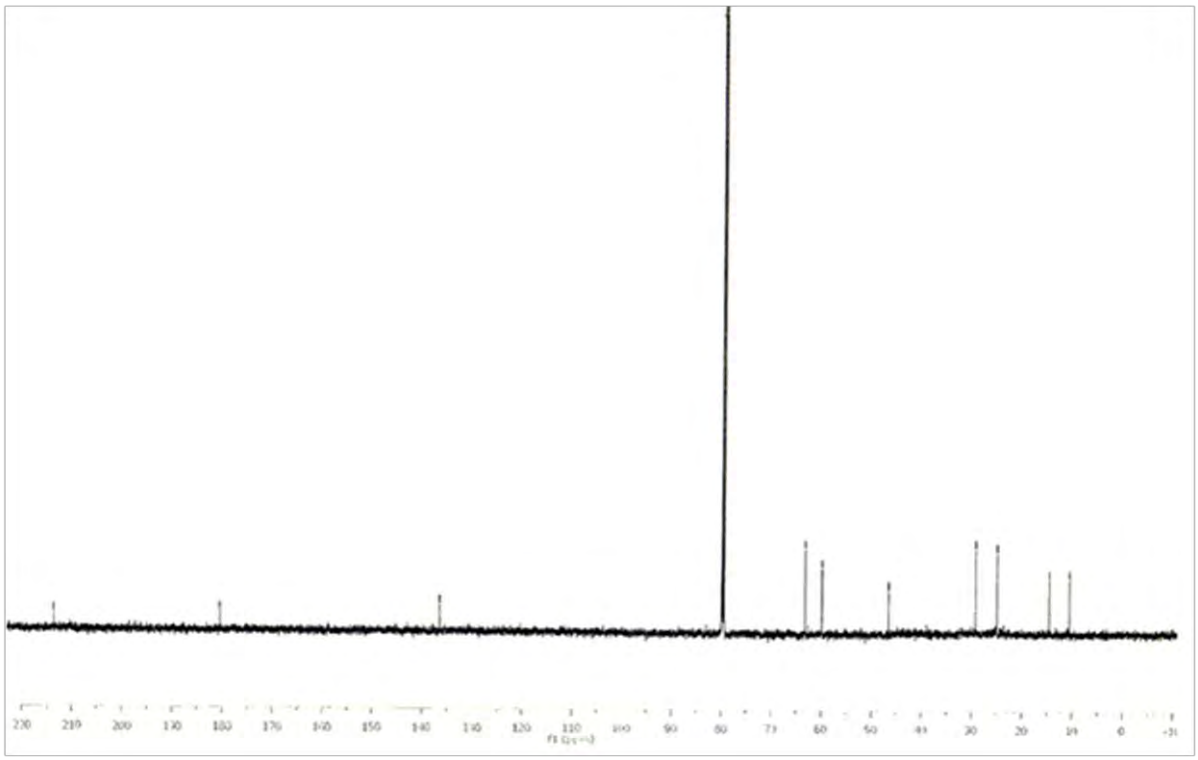


Figure 2: 13C-NMR spectrum of compound 1 (CDCl3, 125 MHz)

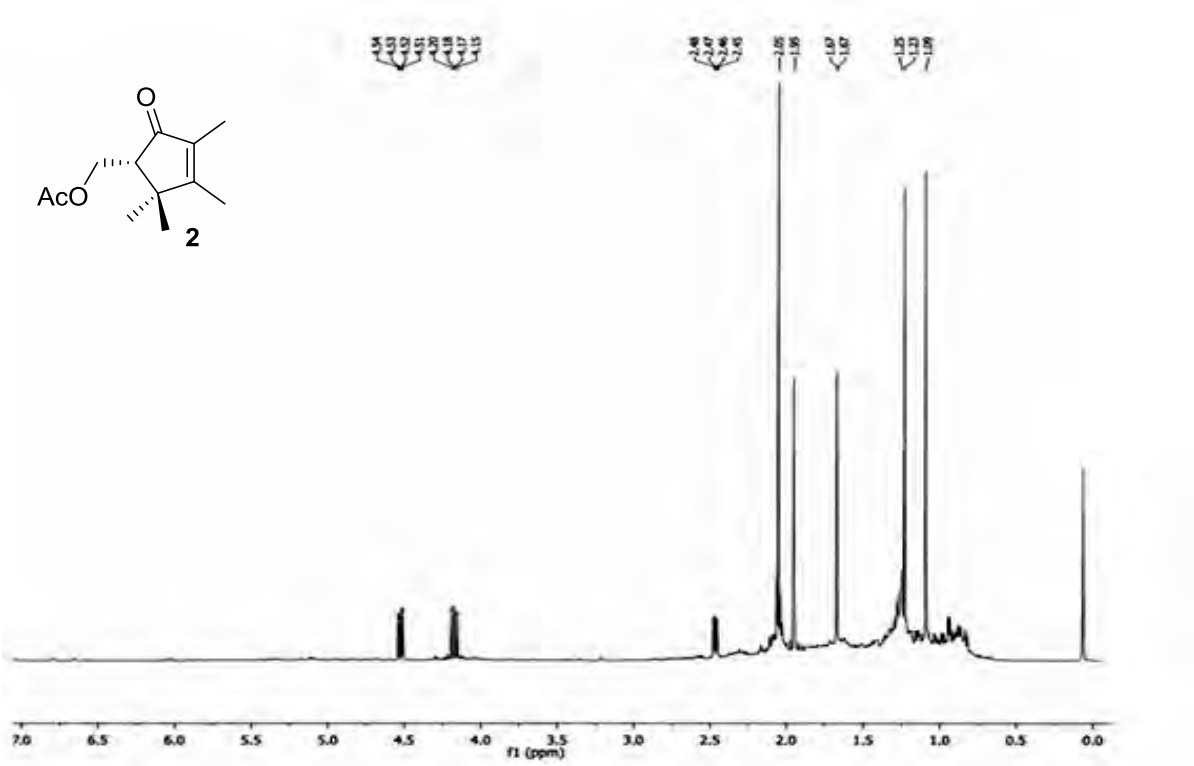


Figure 3: 1H-NMR spectrum of compound 2 (CDCl3, 500 MHz)

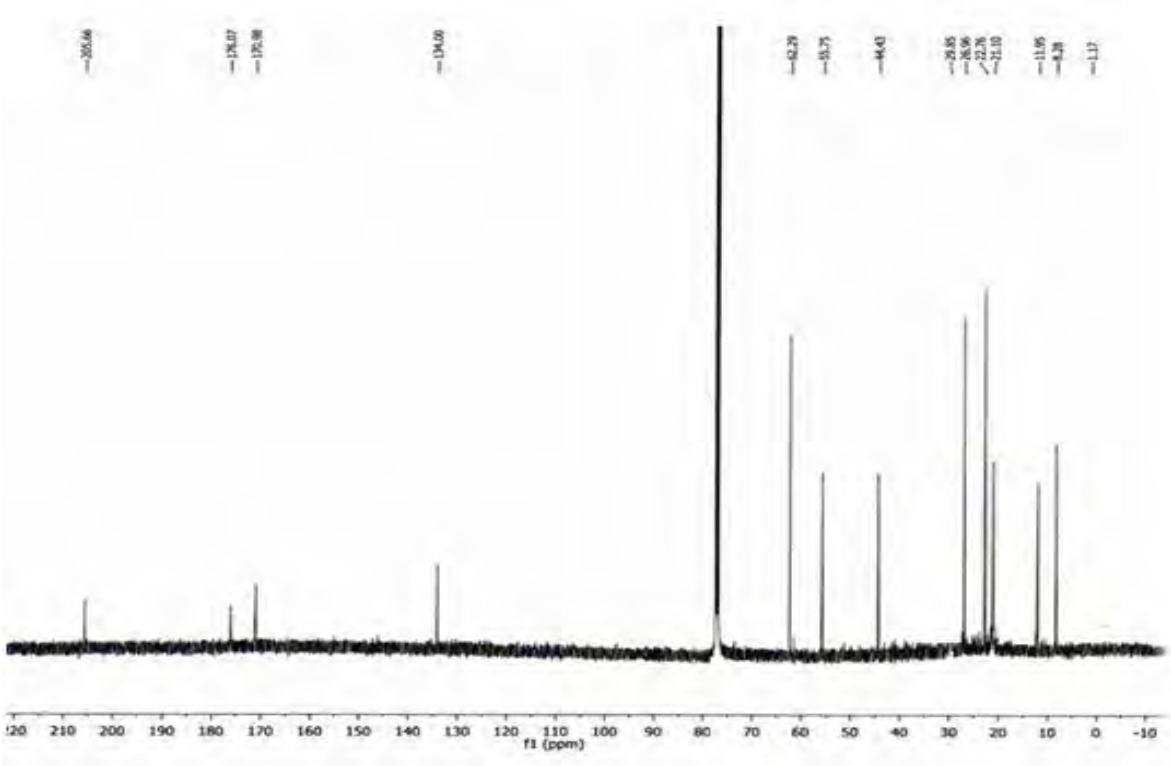


Figure 4: 13C-NMR spectrum of compound 2 (CDCl3, 125 MHz)

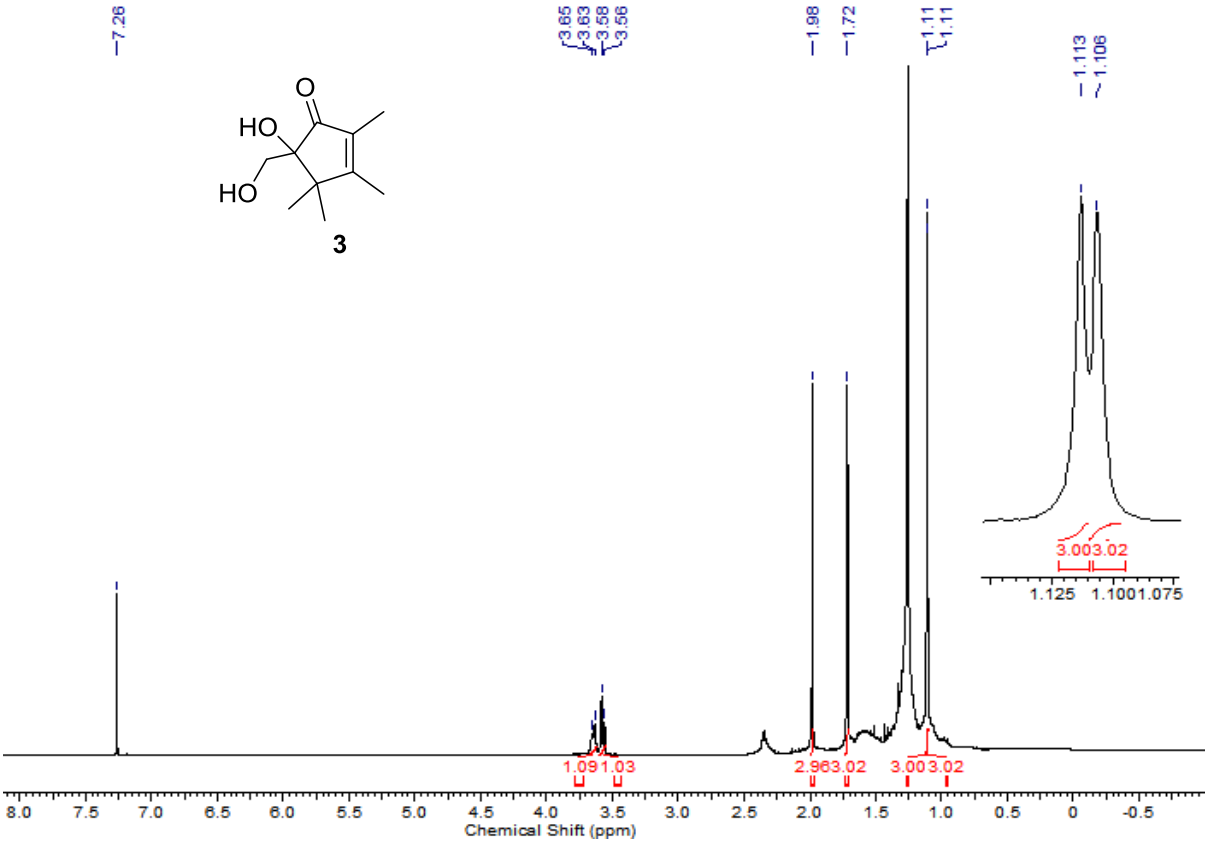


Figure 5: 1H-NMR spectrum of compound 3 (CDCl3, 500 MHz)

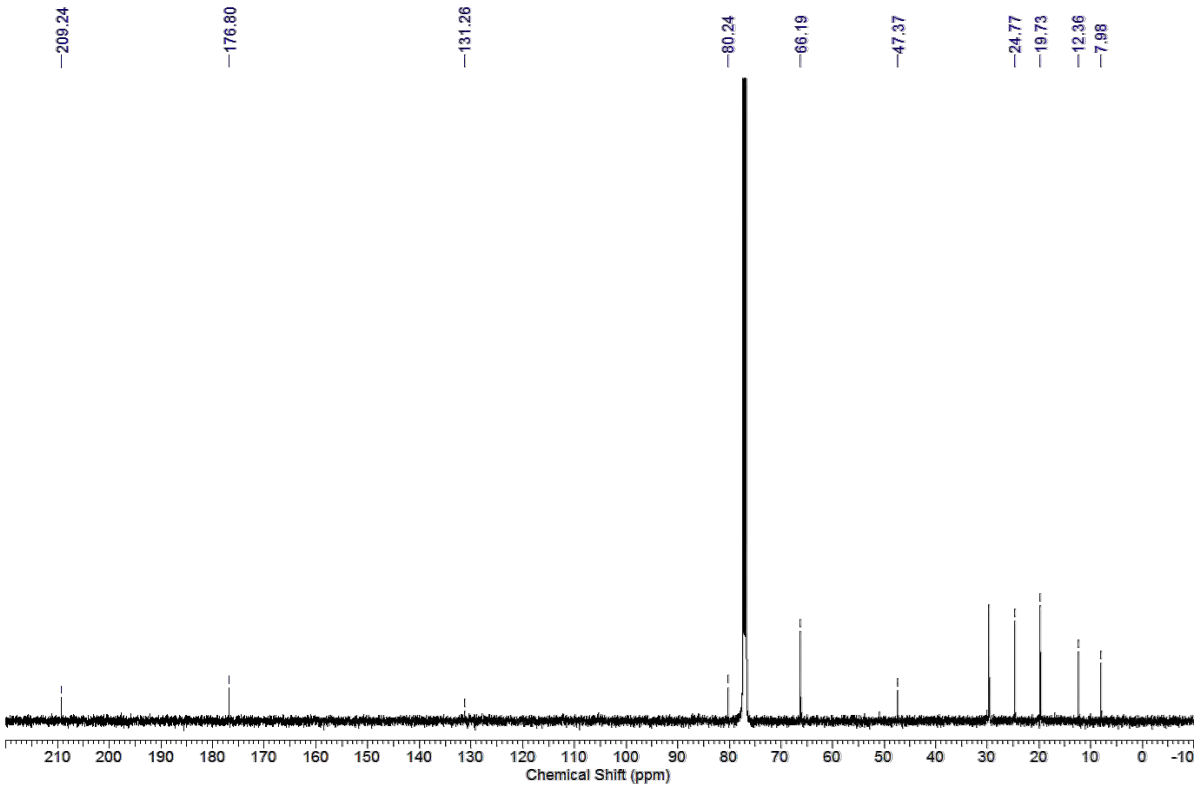


Figure 6: 13C-NMR spectrum of compound 3 (CDCl3, 125 MHz)

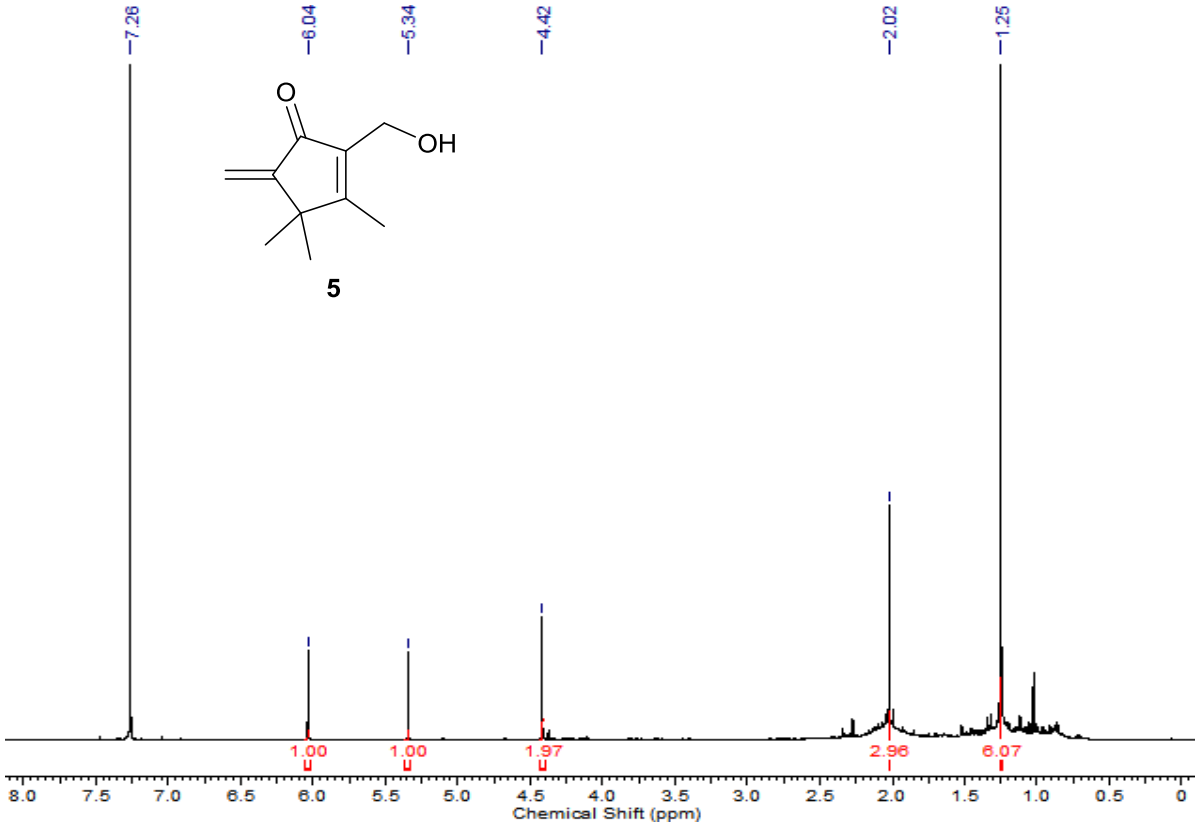


Figure 7: 1H-NMR spectrum of compound 5 (CDCl3, 500 MHz)

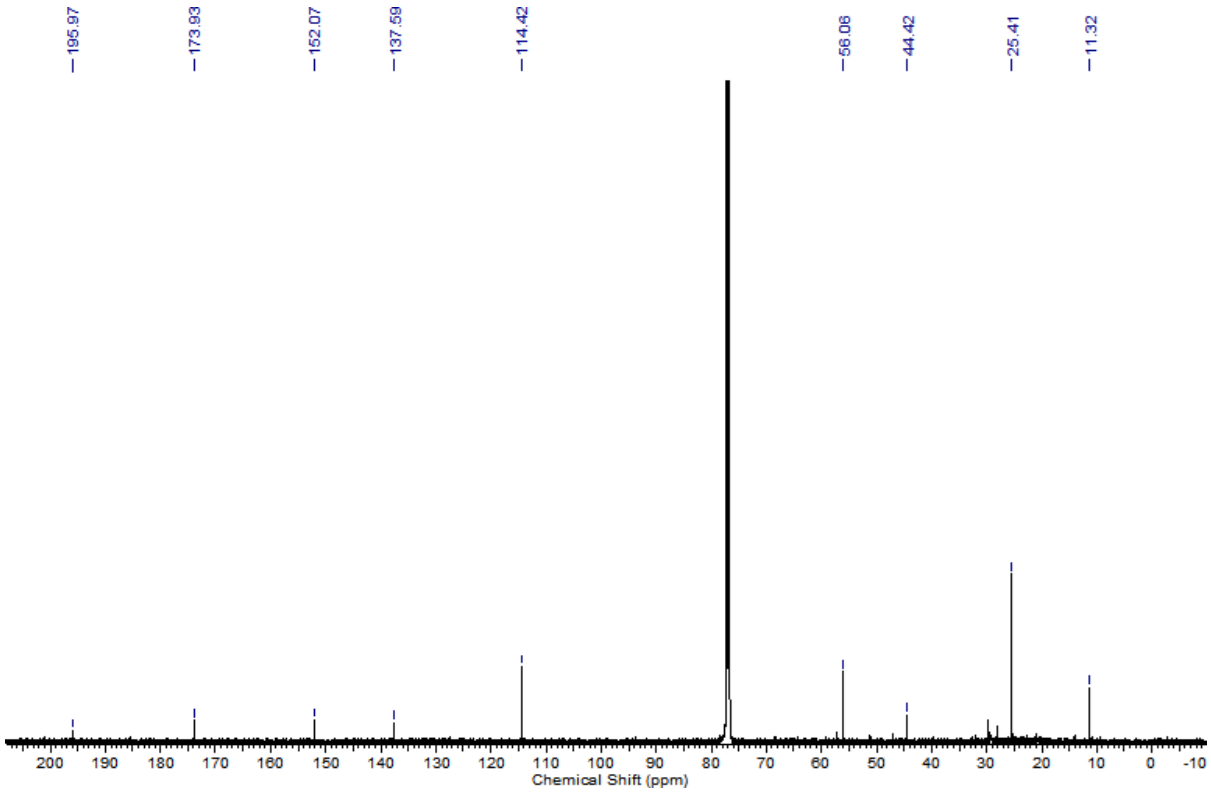


Figure 8: 13C-NMR spectrum of compound 5 (CDCl3, 125 MHz)

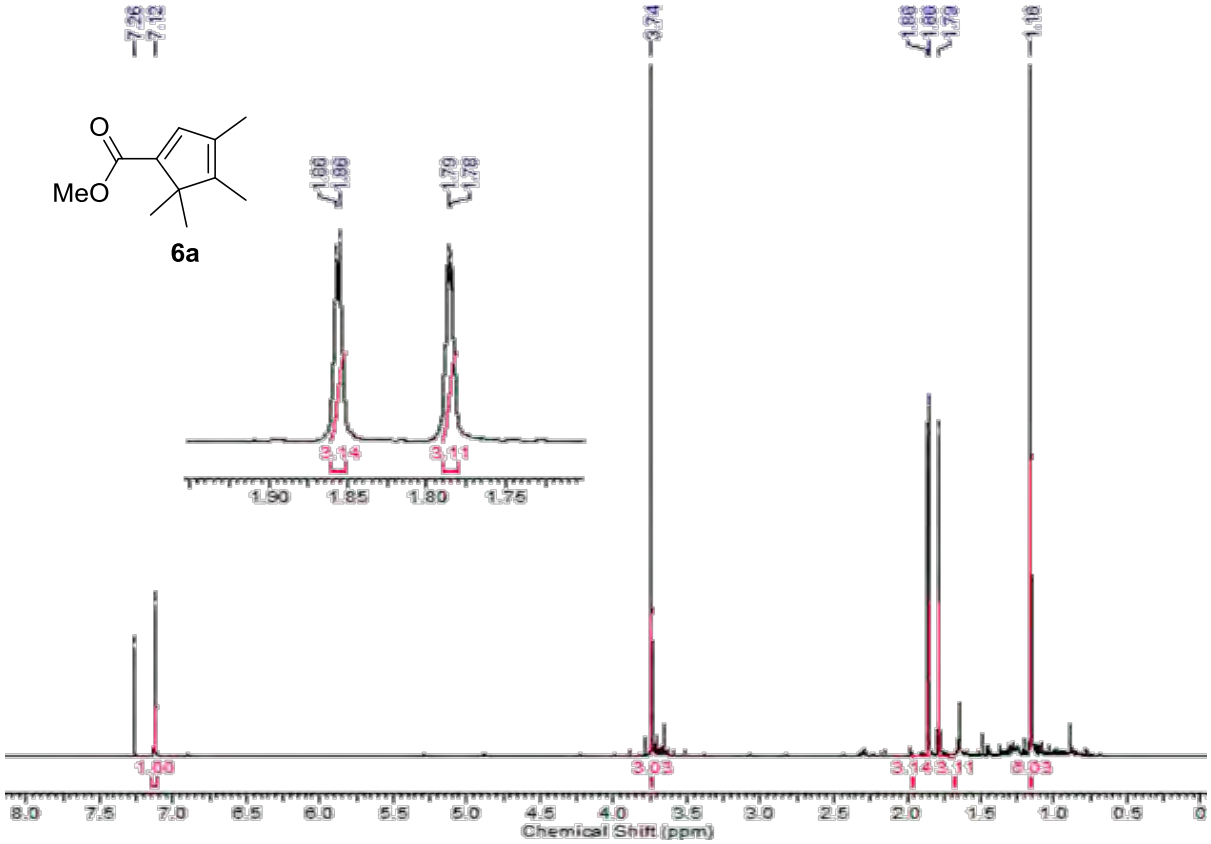


Figure 9: 1H-NMR spectrum of compound 6a (CDCl3, 500 MHz)

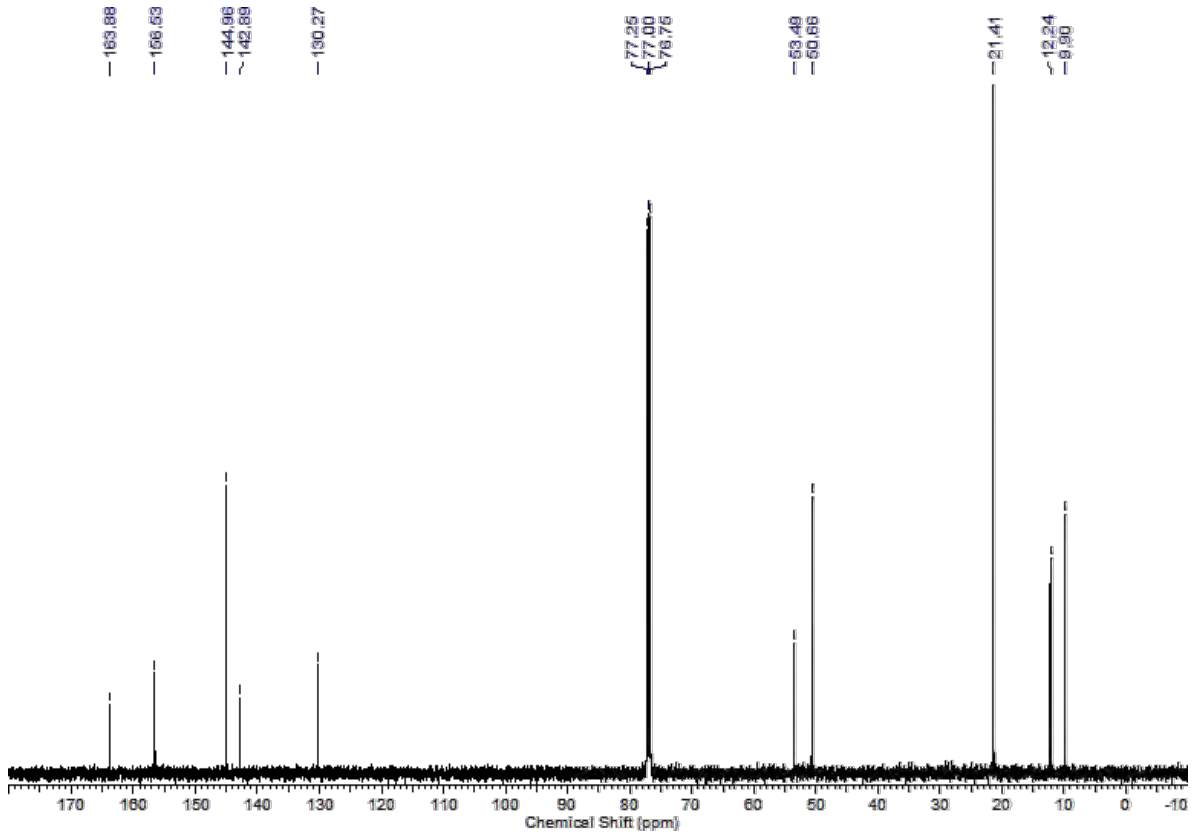


Figure 10: 13C-NMR spectrum of compound 6a (CDCl3, 125 MHz)

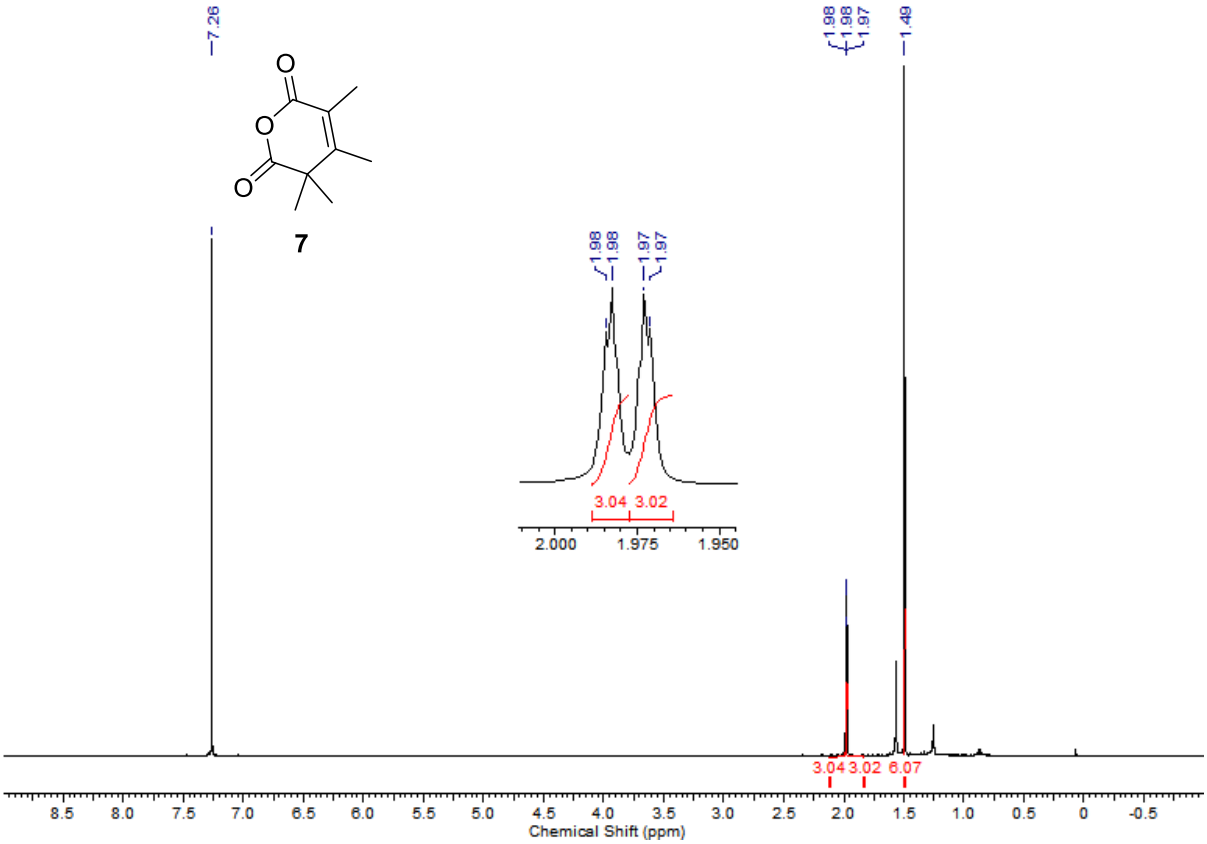


Figure 11: 1H-NMR spectrum of compound 7 (CDCl3, 500 MHz)

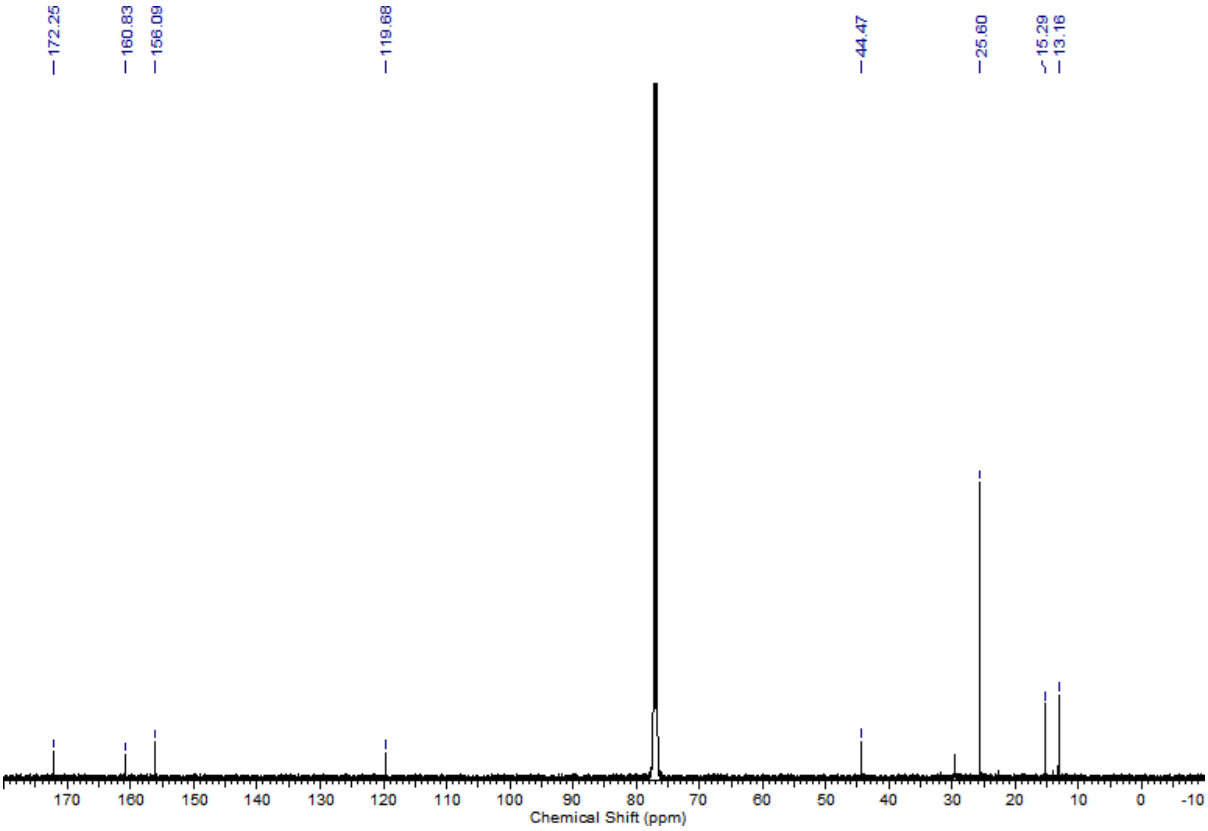


Figure 12: ^{13}C-NMR spectrum of compound **7** ($CDCl_3$, 125 MHz)

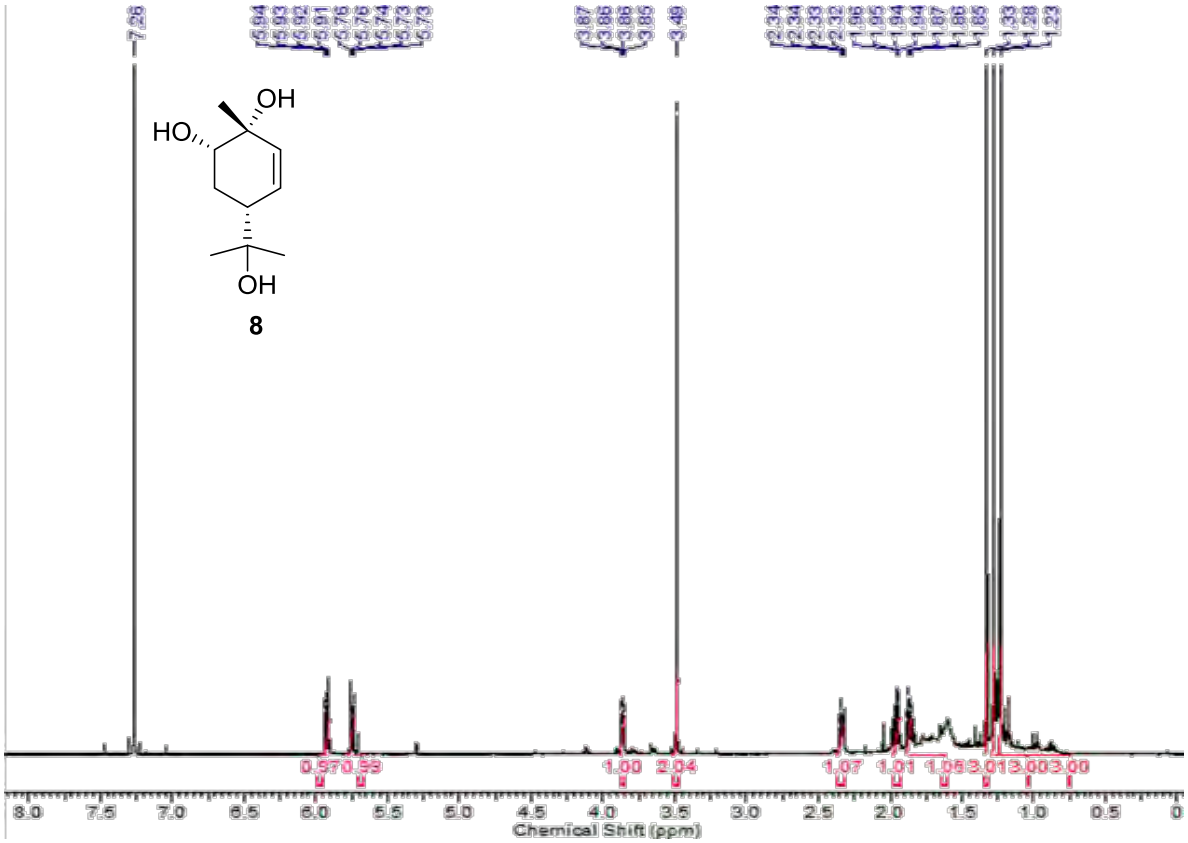


Figure 13: ^{1}H-NMR spectrum of compound **8** ($CDCl_3$, 500 MHz)

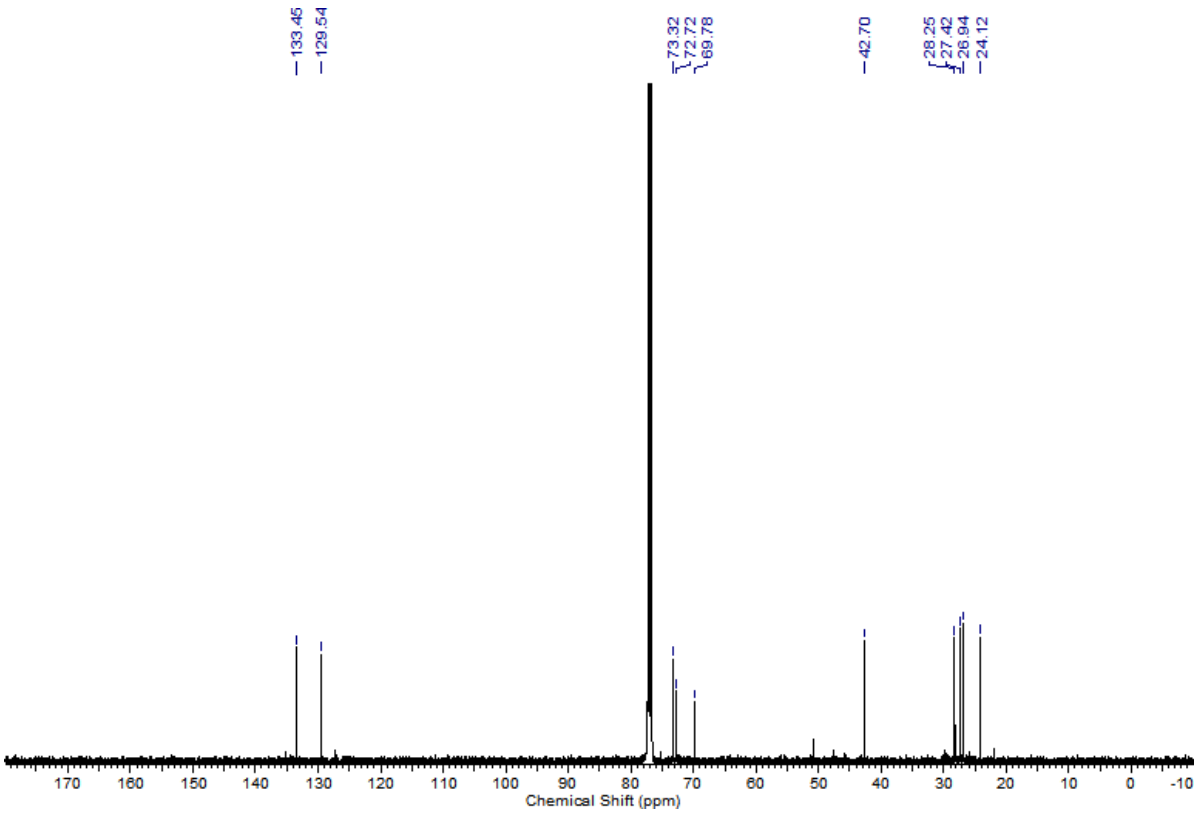


Figure 14: ^{13}C-NMR spectrum of compound **8** ($CDCl_3$, 125 MHz)